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EXPERIMENTAL CELL RESEARCH

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THE EFFECT OF ENUCLEATION ON THE DEVELOPMENT OF SEA URCHIN EGGS

I. ENUCLEATION OF ONE CELL AT THE 2, 4 OR 8 CELL STAGE[1]

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Received April 16, 1952

The object of the present series of papers is to contribute to our knowledge of differentiation by study of the behaviour of enucleated cells. We wish to emphasise that we are using the term differentiation in the sense suggested by Weiss (11) to indicate apparently irreversible processes, as opposed to modulation, which refers to readily reversible changes in cell morphology etc. produced as responses to changes in the environment.

Differentiation presents two apparently distinct problems: (a) the nature of the processes which produce differentiation; (b) the nature of the processes which maintain a constant degree of differentiation. By removal of the nucleus from a cell during early stages of development we expect to obtain information as to the relative importance of nucleus and cytoplasm in producing differentiation. There are three sets of observations possible, which give different types of information. In the first, enucleated material is isolated from nucleated cells: this experiment gives an indication of the extent to which an enucleated cell can develop, in isolation. In the second, enucleated cells are observed when in contact with nucleated cells: these observations give an indication of the extent to which the potentialities of cytoplasm may be supplemented by factors supplied by neighbouring normal cells. In the third, nucleated cells are observed when in contact with enucleated cells: these observations give an indication of the extent to which the normal ability of one cell to influence the differentiation of another cell is retained after enucleation.

MATERIAL AND METHODS

The majority of the experiments were performed on eggs of *Paracentrotus lividus*. Eggs of *Psammechinus miliaris* and of *Echinocardium cordatum* were used for some

[1] This work was reported at the Cytological Congress held at Yale University in September, 1950.

of the enucleation experiments. The results did not differ from those on *Paracentrotus* and will therefore be described together.

The experiments were performed at the Station Biologique, Roscoff, France, during the summers of 1950 and 1951.

The eggs were operated in hanging drops of sea water in a paraffin chamber (2). The de Fonbrune micromanipulator was used. Glass hooks and micro-pipettes were made by means of the de Fonbrune microforge. Eggs and blastomeres were enucleated by aspirating the nucleus with a micro-pipette, the internal diameter of which was somewhat smaller than the diameter of the nucleus. A minimum of cytoplasm was necessarily removed with the nucleus, but it has previously been shown that up to 50 per cent of the central cytoplasm of the egg can be sucked out without impairing the normal development (8). Moreover, eggs enucleated before fertilisation develop into plutei of normal appearance. It can therefore be assumed that any change in the development of the enucleated egg or blastomere is the result of enucleation, not of damage to or loss of cytoplasm.

After the operation the eggs were either kept under observation in the paraffin chamber or placed in individual solid watch glasses containing about 2 ml filtered sea water and picked out for examination at intervals.

Unoperated controls kept under identical conditions were run with each experiment. The temperature was between 18° and 22° C.

Some larvae at different stages were fixed in saturated mercuric chloride solution and stained with borax carmine or with alcoholic cochineal and mounted in balsam. Some larvae were embedded and sectioned.

Enucleation of unfertilised eggs. After aspiration of the nuclei the eggs were placed in solid watch glasses, fertilised and then placed in clean filtered sea water.

Enucleation of 1 cell at the 2, 4 or 8 cell stage. Eggs were fertilised and the fertilisation membranes were removed by squirting the eggs through a narrow pipette into filtered sea water. About 1 hour after fertilisation the eggs were distributed in hanging drops in the paraffin chamber. As soon as the desired stage was reached and the nuclei were clearly visible, the egg was held lightly in a microhook and one of the blastomeres was enucleated.

RESULTS

1. *Enucleation of unfertilised eggs followed by fertilisation.* Of 13 *Paracentrotus* eggs which were enucleated, 12 developed into normal plutei.

2. *Enucleation of 1 blastomere at the 2 cell stage.* This experiment was successfully performed on 47 *Paracentrotus,* 4 *Echinocardium* and 4 *Psammechinus* eggs. In some cases the asters were removed together with the nucleus but in most cases the cytoplasm which gives rise to the asters was left undisturbed. Typical cases of both types will be described in detail.

a) Removal of nucleus only. About 75 minutes after fertilisation (18–20° C) the *Paracentrotus* egg has divided into 2 cells. The nuclei, which are approximately 5 μ in diameter, are reformed in the centre of each cell and are surrounded by somewhat less opaque cytoplasm. At this moment the micropipette was inserted into one cell and brought into contact with the nucleus. Suction was applied sharply and the pipette withdrawn as soon as the nucleus had entered it. Within a few seconds the wound made by the pipette closed up and the enucleated cell became indistinguishable from the normal half, except for the presence of the nucleus in the latter. In the normal blastomere the clear space (centrosphere)[1] which surrounds the nucleus acentrically gradually becomes larger and then the astral rays appear. The asters become more distinct and soon extend towards the periphery of the cell. The nucleus at this stage becomes oval in shape, and seems to be stretched between the asters. The nuclear membrane then disappears and the spindle becomes visible. The chromosomes can rarely be seen at the magnification used (720 ×). The division furrows move inwards from the periphery at right angles to the spindle axis and after completion of the cytoplasmic cleavage the astral rays fade and the nuclei are reformed, still surrounded by the centrospheres.

In the enucleated cell corresponding changes take place: the clear space constituting the centrosphere enlarges and gives rise to asters which grow towards the periphery of the cell. This does not necessarily take place simultaneously with aster formation in the normal half. There is usually a delay and the changes in the enucleated cytoplasm lag behind those in the normal cells. In the majority of experiments no cleavage furrows were observed in enucleated cells during the first 5–7 hours but the asters multiply at regular intervals until between 16 and 64 are present (Figs. 1 and 2). During this time the normal cells continue to divide and form a half blastula which tends to grow over the as yet undivided enucleated blastomere.

In some cases, however, cleavage furrows appeared in the enucleated cell rather earlier, i.e. 2–3 hours after enucleation. The furrows did not always extend throughout the cytoplasm but were incomplete. In this manner 2–8 "cells" of irregular sizes and shapes would be formed (Figs. 2 b, c). An interesting feature of these incomplete cleavage furrows is that they were temporary: for periods of several hours (usually 3–6 hours after enucleation) these furrows would be formed and reformed in various directions while the asters increased in number.

About 6–8 hours after enucleation the asters became less distinct and

[1] For nomenclature see Lorch (10).

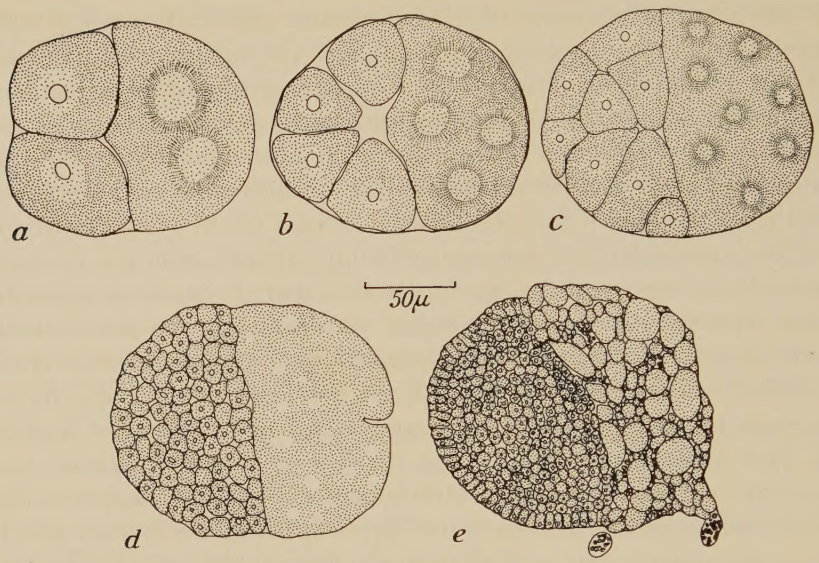

Fig. 1.

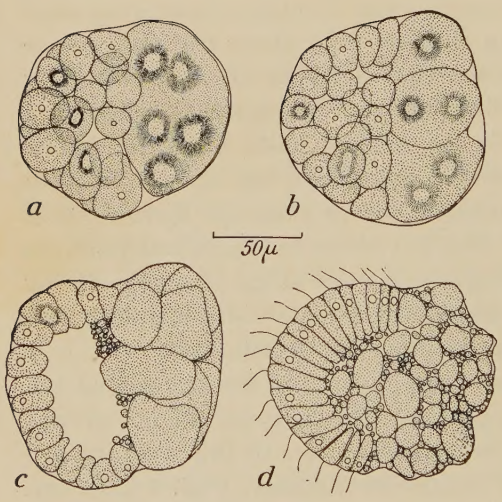

Fig. 2.

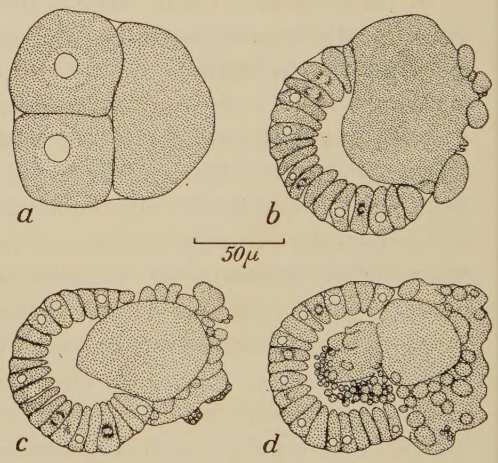

Fig. 3.

Textfigures 1–3 are camera lucida drawings of living cleavage stages of *Paracentrotus lividus*. The enucleated blastomere is on the *right* side. Textfigures 4–6 are drawn from whole mounts of stained embryos.

Fig. 1 a–e. Successive stages in the development of a slightly compressed egg in a hanging drop under paraffin. One blastomere was enucleated at the 2 cell stage. a. 40 mins after enucleation. The normal blastomere has divided, the enucleated half has formed 2 asters. b. 80 mins after enucleation. The normal half has 4 blastomeres. The enucleated half has 4 asters. c. 2½ hours

finally the cytoplasm appeared homogeneous. This took place in all cases, whether division furrows were present or not. Shortly after the disappearance of the asters the cell divided into many spheroidal cells of irregular sizes. This division took place fairly quickly, thus within an hour of the disappearance of the asters the enucleated blastomere had changed into a mass of these spheroidal cells (Figs. 1 e and 2 d). This process may be termed "pathological cleavage" to distinguish it from normal cytoplasmic division which is a much slower and more regular process and is correlated with nuclear division and the formation of asters. The products of this abnormal cleavage were roughly spherical masses of cytoplasm, each surrounded by an intact plasma membrane, and may therefore be termed "cells". The normal half of the egg had by this time grown over all or part of the mass of enucleated "cells" which thus became enclosed in the blastocoele (Figs. 2 d, 4 and 7).

Some of the enucleated material was usually left outside the blastula and stuck to its exterior surface for some time, after which it fell off and could be seen lying intact at the bottom of the dish for 2–3 days before it disintegrated.

In some experiments cytolysis of damaged cells occurred. It is important to be able to distinguish accurately between cytolysis and true division of cytoplasm. A good method of doing this is to use a very dilute solution of trypan blue in sea water. Normal cells, and enucleated but otherwise undamaged cells, are practically impermeable to trypan blue, and are not stained even after two or three days. On the other hand, cytolysing cells

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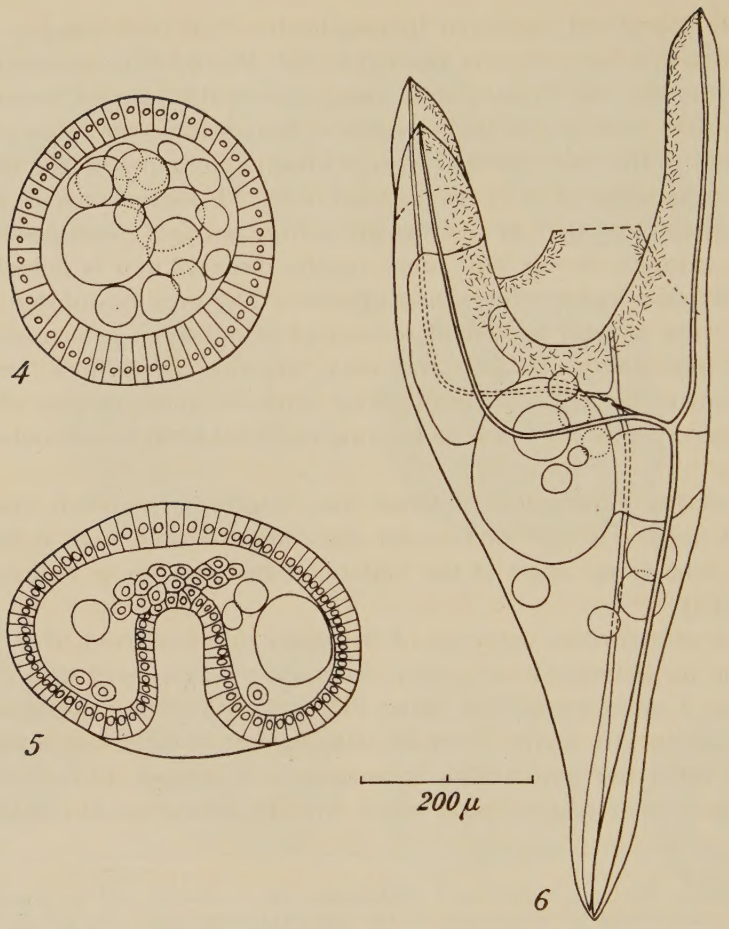
after enucleation. The normal half has 8 blastomeres. The enucleated half has 8 asters. All cells are in one plane owing to compression of the egg. d. $6\frac{1}{4}$ hours after enucleation. The normal half is forming a half blastula (surface view shown). The enucleated half has many faint asters (clear spaces) and a small incomplete division furrow. e. $10\frac{1}{4}$ hours after enucleation. Cleavage continues in the normal half (surface view of $\frac{1}{2}$ blastula shown). The enucleated half has divided into irregular cells. No asters seen.

Fig. 2 a–d. Successive stages in the development of an egg. Not compressed. One blastomere was enucleated at the 2 cell stage. a. $4\frac{1}{2}$ hours after enucleation. The normal half has about 32 cells, the enucleated half has 8 asters (only 5 in plane of drawing). b. 10 minutes later: the enucleated half has divided into 3 irregular cells. c. 6 hours after enucleation. The normal half has formed a $\frac{1}{2}$ blastula, the enucleated half has divided into irregular cells in which no asters are seen. Optical section. d. 10 hours after enucleation. Optical section through swimming blastula. The nucleated cells enclose enucleated cells.

Fig. 3 a–d. Successive stages in development of an egg in which the *asters* as well as the nucleus were removed from one blastomere at the 2 cell stage. a. 35 minutes after operation. The normal blastomere has divided. The operated blastomere appears structureless. b. $4\frac{1}{2}$ hours after operation. The normal half is forming a $\frac{1}{2}$ blastula. The operated half shows the first signs of division at the periphery. Optical section. c. 7 hours after operation. Division of the operated half into irregular cells continues. d. 10 minutes later: nearly the whole operated half is divided into irregular cells.



Figs. 4, 5 and 6. Camera lucida drawings of optical sections through whole mounts of *Paracentrotus lividus* larvae stained with Borax Carmine. Fig. 4. One cell enucleated at 2 cell stage. Fixed 17 hours later at blastula stage. Note the spheroidal anucleate cells in the blastocoele. Fig. 5. One cell enucleated at 8 cell stage. Fixed 22 hours later at gastrula stage. Large anucleate cells as well as primary mesenchyme cells are seen in the cavity. Fig. 6. One cell enucleated at 4 cell stage. Fixed 57 hours later as fully developed pluteus. Note large anucleate cells in the cavity. The individual cells of the ectoderm, gut etc. are not drawn.

have lost the specific permeability characteristic of living cells, and quickly become deeply stained by trypan blue.

Enucleated cells, and cells derived from division of enucleated cells, whether in the blastula cavity or otherwise, did not stain with trypan blue over a period of two to three days. They are thus charcteristically different from debris and "dead" cells.

Optically these cells appeared evenly granular, resembling the cytoplasm of normal nucleated cells during interphase. Once the enucleated half had divided into this mass of cells no further division seemed to take place. The cells of the normal half continued to divide and differentiate until more or less normal plutei were formed. The larvae were not always quite perfect: the mass of enucleated material seemed to disturb gastrulation in some cases and in others the bilateral symmetry of the pluteus was distorted. The more enucleated cells were enclosed in the cavity, the less perfect was the resulting larva. On the other hand, material attached to the outside of the blastula disturbed the swimming, and such "weighted" larvae remained at the bottom of the dish until the enucleated cells had fallen off. Thus the enucleated cells can affect the development of the larva in various ways which we suspect to be passive. But in no case were the enucleated cells observed to differentiate nor were they ever incorporated into the blastula wall or into any organ of the pluteus. Only the nucleated cells derived by division of the unoperated blastomere formed the larvae which were about half the normal size. Some blastulae were bigger than half size owing to the distension of the cavity by enucleated material. The fate of the enucleated cells was followed in the living larvae and confirmed by study of whole mounts of stained preparations (Figs. 7 and 8).

b) *Removal of nucleus and asters.* If the centrosphere surrounding the nucleus is sucked out together with the nucleus then no asters are formed. The enucleated cell remains unchanged for 5–7 hours (fig. 3 a) after which division into small spherical cells takes place in exactly the same way as if the asters had been present (Fig. 3 b–d).

The question of the possible role of asters in cytoplasmic division is discussed in another paper (10).

3. *Enucleation of 1 cell at the 4 or 8 cell stage.* One cell in four was enucleated in 36 *Paracentrotus* and 6 *Psammechinus* embryos and one cell in eight in 26 *Paracentrotus* and 6 *Psammechinus* embryos. The results were essentially the same as those recorded in the previous section. The enucleated blastomere divided into a number of spheroidal cells of irregular sizes. These were found in the cavity of the developing larva and were never seen to differentiate nor form any organ. Disturbances of development were very rare in experiments where one of four cells was enucleated and absent when only one of eight cells was operated. This is presumably due to the proportionally smaller volume of enucleated material which has to be dealt with by the normal cells. Figs 5, 6 and 9 show larvae from eggs in which one cell was enucleated at the 4 or 8 cell stage.

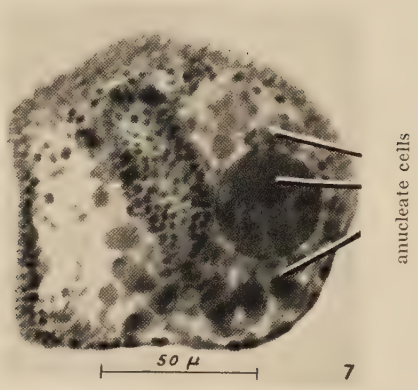
DISCUSSION

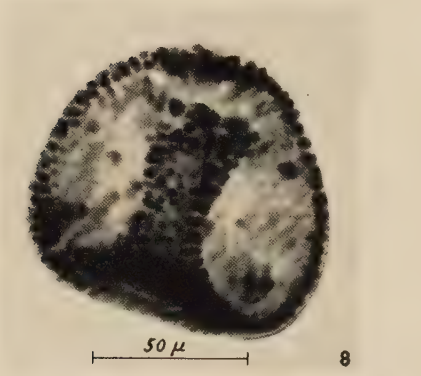
The main results which have been obtained in the experiments described above are: 1. Enucleated cells retain the ability to divide. 2. The cleavage of enucleated cells is abnormal. 3. The cleavage of enucleated cells is not a precursor of cytolysis, nor is the permeability of the daughter cells abnormal. 4. Even when in close contact with nucleated cells, enucleated cells show no sign of differentiation. 5. When studied up to the pluteus stage, no evidence could be obtained that enucleated cells are ever incorporated in tissues.

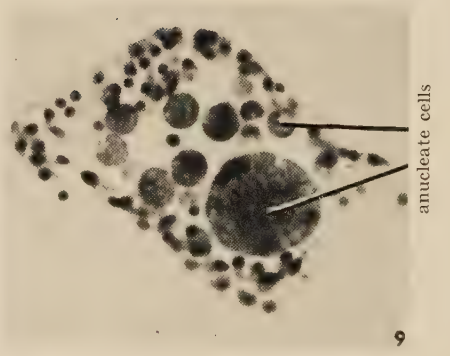
The first, fourth and fifth results have been reported by many other observers, e.g. Ziegler (14), Wilson (12), Yatsu (13), Jollos and Peterfi (9), Fankhauser (3, 4), Harvey (5, 6, 7), Briggs, Green and King (1). But evaluation of the earlier observations has been difficult because of the pathological nature of the material used. This consisted either of occasional ova which behaved quite differently from normal ova, or of material which had been subjected to violent experimental procedures. For example, Ziegler described an egg of *Echinus microtuberculatus* where, after fertilisation, no nuclear material passed into one of the two first cleavage cells. Yatsu studied egg fragments, and Harvey studied half-eggs obtained by centrifugation. Obviously negative results obtained on such pathological material are difficult to evaluate.

We are confident that this criticism cannot be made of our experiments, for our control experiments make it clear that cells treated by our technique retain their full capacity for normal development. These control experiments were of two types. In the first type the nucleus was not removed, but up to 50 per cent of the cytoplasm was sucked out. These cells developed quite normally, though of course into smaller larvae than usual (8). In the experiments reported now, only a very small amount of cytoplasm was removed with the nucleus: consequently the failure of our enucleated cells to differentiate cannot be attributed to quantitative loss of cytoplasm, since the loss of much larger quantities of cytoplasm from nucleated cells causes no abnormality. It can still be argued that the failure to differentiate may be due to the loss of some special type of cytoplasm closely adjacent to the nuclear membrane. This difficulty is overcome to some extent by our second type of control experiment. In this, normal ova were enucleated and then fertilised: development was normal. It is still possible that a source of the supposed special cytoplasm was the sperm, and we cannot at present counter this criticism.

To sum up, we are confident that the results 1–5 are not attributable to the use of pathological material, nor to damage caused by our procedure.







Figs. 7, 8 and 9. Unretouched photomicrographs of stained preparations. Fig. 7. *Paracentrotus lividus.* One cell enucleated at 2 cell stage. Fixed 48 hours later at "prism" stage. Whole mount stained with alcoholic cochineal. Note spheroidal (anucleate) cells in cavity. Fig. 8. Similar preparation to fig. 7, showing a normal gastrula. The small cells seen in the cavity are primary and secondary mesenchyme and are easily distinguished from the large spheroidal (anucleate) cells depicted in fig. 7. Fig. 9. *Psammechinus miliaris.* One cell enucleated at 4 cell stage. Fixed 42 hours later (Early pluteus). Decalcified, longitudinal section, 10 μ, stained haematoxylin and eosin. Some of the large spheroidal (anucleate) cells are seen.

We cannot, however, be certain that the abnormalities observed are entirely due to loss of a nucleus, since it is possible that when a nucleus is removed some special cytoplasm adjacent to the nucleus is also removed.

SUMMARY

Enucleated blastomeres left in contact with one, three or seven normal blastomeres did not play any part in the development of the larva, except in so far as purely mechanical distortion was produced.

After an initial delay, during which asters and incomplete division furrows appeared and disappeared again, the enucleated cells divided rapidly into a number of spheroidal cells of various sizes. The final diameter of these cells ranged from 3 to 50 μ. The cells remained optically similar to nucleated cells of the same size and retained their semi-permeability for 3 days or more.

Enucleated cells were never seen to differentiate or participate in the formation of tissues or organs.

The presence or absence of asters in the early stages made no difference to the final fate of the enucleated cells.

Thus it seems that the nucleus is essential for differentiation, though not for cell division. The proximity of normally differentiating cells does not induce similar changes in the enucleated cells.

We should like to thank Professor G. Teissier, Professor P. Drach, Dr. Ch. Bocquet, Dr. C. Levi and the staff of the Station Biologique, Roscoff, France, for their co-operation in obtaining the necessary material. Part of the expenses was defrayed by a grant from the Royal Society to Professor J. F. Danielli, and from the Swedish Research Council to Professor Hörstadius, for which we should like to express our thanks. We are also grateful to Mr. F. S. Russell, F.R.S., and the staff of the Marine Biological Laboratory, Plymouth, where preliminary experiments were carried out in 1949.

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II. ENUCLEATION OF ANIMAL OR VEGETAL HALVES

S. HÖRSTADIUS, I. JOAN LORCH, and J. F. DANIELLI

The development of isolated animal and vegetal halves of sea urchin eggs has been closely studied (3). Such halves are produced by cutting between the macromeres and mesomeres at the 16 or 32 cell stage (Fig. 1). It has been shown that most animal halves possess a more or less enlarged apical tuft and neither ciliated band nor stomodaeum. They never gastrulate nor

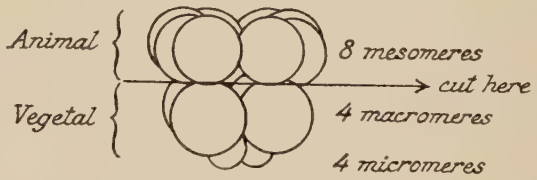

Fig. 1. Diagram illustrating the preparation of control animal and vegetal halves by cutting at the 16 cell stage.

do they form a skeleton. These characteristics are usually referred to as animalisation. Animal halves have been classified according to the extension of their apical tuft. Animal halves from eggs of different females differ in the extent to which the tuft is developed but halves from the same batch are as a rule of more or less similar type. Similarly the vegetal halves exhibit different types from dwarf plutei to larvae of ovoid shape without arms, with a poor and irregular skeleton and often without apical tuft and mouth. They may also form exogastrulae. These characteristics are usually referred to as vegetalisation. When animal and vegetal halves from the same egg are reared it is found that, as a rule, the "most animal" halves correspond to the most typically developed vegetal halves and vice versa.

In the intact egg the animal and vegetal gradients react on each other. Vegetal material induces formation of stomodaeum and ciliated band. The animal half is often unable to form these structures by itself. Micromeres implanted into animal halves can induce an archenteron. It may then be asked whether (a) the presence of vegetal *cytoplasm* alone would have a similar inductive action, and (b) whether the enucleated vegetal cells themselves are able to divide and differentiate while in contact with the normal animal half. Conversely: does the presence of enucleated animal cells induce a more normal development in the contiguous vegetal half? That cells enucleated immediately after the first, second or third division do not differentiate or participate in tissue formation was shown in the first part of this

work (7). Owing to the fact that vegetal halves from certain batches of eggs are able to form small plutei it is important to obtain series with vegetal halves of pronounced vegetal type for this experiment, so that any effect which the enucleated animal half may have can be clearly assessed by comparison with the isolated control halves. It is, at the beginning of an experiment, impossible to know which type of halves a batch of eggs will give. The effect of enucleated vegetal halves on the intact animal halves should be easier to assess since isolated animal halves, although showing some variation, never gastrulate nor form skeletons, whereas animal halves with implanted micromeres develop into plutei. The micromeres themselves give skeleton-forming cells and induce an archenteron. Would vegetal cytoplasm without nuclei have a similar effect?

This problem has previously been attacked by Hörstadius ((2) p. 358) who implanted vegetal cytoplasm of fertilized eggs into animal halves. No change of differentiation was achieved. This may have been due to the fact that the cytoplasm was not incorporated in the blastula wall, as implanted micromeres are, but remained lying free in the blastocoele. In order to bring about a closer contact, Hörstadius, in other experiments (unpublished), put a number of fragments of vegetal cytoplasm (isolated from fertilized eggs by aid of the pigment band as orientation) at the bottom of a small excavation in a celluloid plate and then pressed the edges of isolated animal halves against them. Animal halves were also treated with extract of micromeres prepared in a capillary tube. Both kinds of experiments were negative.

The following experiments aimed at testing the effect of enucleated cells that had never been removed from their neighbours so that the contact between the normal and the enucleated cells was undisturbed.

METHODS

The experiments were performed during the summers of 1950 and 1951 at the Station Biologique, Roscoff, France. Eggs of *Paracentrotus lividus* were used. The general technique of manipulation of the eggs, enucleation of blastomeres and rearing of larvae is described in our first paper (7). In the present experiments special care has to be taken in order to ensure the enucleation of 4 animal or 4 vegetal blastomeres and not, say, 3 animal and 1 vegetal cell, or a meridional half.

The following method was used: demembranised eggs at the 4 cell stage were distributed in hanging drops in the paraffin chamber. The drops must be fairly deep (high contact angle between the sea water and coverslip). If the 4 cell stages are compressed in shallow drops then, after the following division, all 8 cells come to lie in one plane, whereas if the drop is fairly convex then the animal and vegetal halves will lie in different planes, forming a cube of 8 cells. Now the upper ring of

4 cells (i.e. the layer closest to the coverslip) consists of *either* 4 animal *or* 4 vegetal cells. In some cases these can be distinguished by their size; the vegetal cells are often smaller than the animal cells. A much safer method of identification is to await the formation of asters just prior to the division of the 8 cells into 16 cells: the plane of the asters in a vegetal cell is nearly parallel to the egg axis whereas in an animal cell it is parallel to the equatorial plane. Since the asters are formed just prior to the dissolution of the nuclear membranes it is possible to remove the nuclei of the desired layer of cells (the asters are left intact).

However, in practice it is always simpler to remove the nuclei of the upper ring of 4 cells and to identify the operated layer after division of the non-operated cells: if the latter divide unevenly into 4 macromeres and 4 micromeres then the vegetal half must be intact, i.e. the animal half was enucleated. However, if the unoperated cells divide evenly then they are most likely[1] animal cells, i.e. the vegetal half was enucleated.

After the operation the eggs were observed, first in the drops and later in individual watch glasses. The following controls were done:

(a) Enucleation of meridional halves: 2 animal and 2 vegetal cells were enucleated. These usually developed into normal, though small, plutei and will not be further described.

(b) Preparation of animal and vegetal halves: these were isolated with glass needles in calcium-free sea water at the 16 or 32 cell stage. Controls were always taken from the same batch as the operated eggs. All larvae were examined under high power and drawn at the blastula stage and again the following day.

In some cases the enucleated cells were removed by means of a glass needle after they had been in contact with the normal half for several hours.

RESULTS

In July 1950 experiments were done on eggs from 2 females and a total of 6 animal halves (vegetal cells enucleated) and 8 vegetal halves (animal cells enucleated) developed into free swimming larvae. These experiments were continued in June and July 1951 when 6 batches of eggs were operated, resulting in a further 31 animal and 29 vegetal larvae.

a) *Behaviour of the enucleated cells.* The fate of the 4 enucleated cells at the 8 cell stage does not differ from that already described for cases in which only one cell is enucleated (7). The cells usually stayed intact for about 5 hours and then rapidly divided into a number of more or less spheroidal cells of various sizes. By this time the normal cells had formed a half blastula which then often grew over part of the enucleated material enclosing it in the cavity. Typical stages of this process are illustrated in Fig. 2 a–d. The remaining cells fell off and eventually disintegrated. In no case was differentia-

[1] One cannot be absolutely sure of this since the vegetal halves sometimes fail to form micromeres, i.e. divide evenly (1).

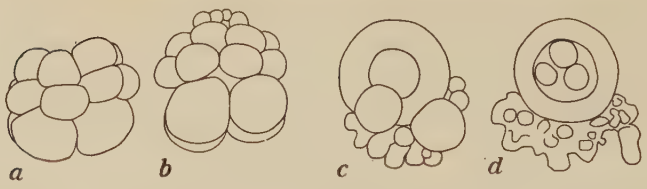

Fig. 2. Typical stages in the early development of embryos which have 4 enucleated cells. Camera lucida drawings of living specimens. The enucleated cells are at the bottom. (a) About 45 minutes after enucleation of the vegetal cells. (b) About 2 hours after enucleation of the animal cells. The 4 enucleated cells are still intact. (c) About 7 hours after enucleation. The enucleated cells have divided irregularly and are outside the blastula wall. (d) A different larva, 6 hours after enucleation. Here the enucleated cells are partly inside, partly outside the blastula.

tion of enucleated cells observed. In the first hour after enucleation asters could often be seen in the cells. It is interesting to note that immediately after enucleation of, say, four vegetal cells, the asters were formed in the normal plane and position for the formation of micromeres and macromeres but no division furrows were seen at the normal time. Similarly in the enucleated animal cells asters were formed in the normal radial position.

b) *Development of the operated embryos.* Free swimming larvae were formed in most cases where 4 out of 8 cells were enucleated. It must be noted that only the *nucleated* cells differentiated and gave rise to the various structures. Thus in cases where the development was "more normal" than would be expected from, say, 4 animal cells alone this must be due to an influence of the vegetal cytoplasm on the animal cells and not to participation of the anucleate vegetal cells themselves.

(I.) *1950 Experiments*

a) *Animal halves with enucleated vegetal cells.* In 1950 two series of experiments were made. In each series all eggs used were taken from the same female. Six animal halves with enucleated vegetal cells developed into free swimming larvae, 3 in each series. They resembled the control halves as shown in the following table. The extension of the apical tuft is indicated by the figures 1/2, 1/3, 1/4, 1/6, referring to how much of the surface of the blastula is covered with long stiff cilia. A normal tuft would be designated as 1/8. The types of fully differentiated blastulae are: A, uniformly ciliated blastula; B, blastula with a ciliated field and the other part of the wall consisting of pavement epithelium; C, blastula with ciliated band; D, blastula with ciliated band and stomodaeum.

The operated cases are very few but it is striking how they coincide in type

TABLE I

Animal halves with enucleated vegetal halves. Table showing extent to which the surface of the blastulae formed from animal halves was covered by cilia (1/2–1/6), and the later differentiation (A–D).

	1/2	1/3	1/4	1/6
Exp. A			2	1
Control to A		3	4	2
Exp. B		3		
Control to B	5	11	2	
	A	B	C	D
Exp. A		1	1	1
Control to B	1	2	3	2
Exp. A		1		
Control to B		12	2	

with the majority of the controls in each series. The blastocoele of the operated halves was more or less filled with parts of the enucleated (vegetal) cells, which had divided in the manner found typical for anucleate cells (Fig. 2). Although the enucleated vegetal cells after the operation still had the normal contact with the animal half and although parts of the cells were enclosed in the blastula, they did not cause a more harmonic differentiation of the intact animal cells.

b) *Vegetal halves with enucleated animal cells.* When judging the results of vegetal halves in contact with enucleated animal halves we have to compare the operated larvae of each series with the control halves from the same batch of eggs. Fig. 3 shows in the upper row (a_1–a_5) all the 5 control vegetal halves and the lower row the 4 operated specimens. One of these larvae (d) resembles the controls but the other three deviate in a remarkable way. They resemble normal plutei, except that the most animal part, the oral lobe with the oral arms, is not quite typically developed. They all have ciliated bands and stomodaeums. This indicates that the animal properties in these larvae are much stronger than in the other 6 larvae in Fig. 3. The very surprising conclusion would be that this animal influence has been brought about by the anucleate animal cells.

The 18 controls of the other series (Fig. 4) are not of such unitary type as those in Fig. 3. In 3 cases the vegetal principle is so dominating that they have formed exogastrulae. 4 or 5 more or less resemble plutei but have short

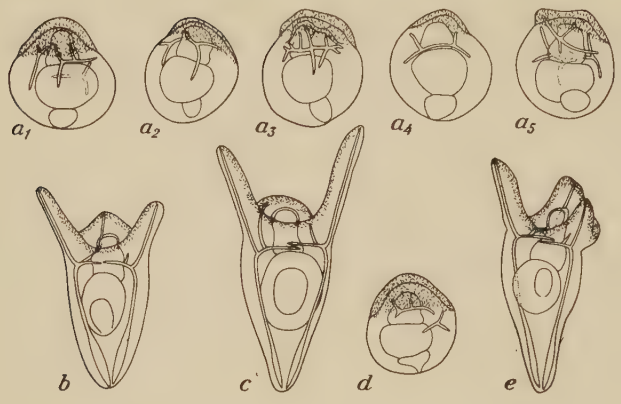

Fig. 3. Enucleation of the animal half. 1950 experiments. First batch. (18/7 on Table III.) This and all subsequent figures are camera lucida drawings of specimens freshly killed with formalin vapour. a_1–a_5 The control vegetal halves. b–e Vegetal halves with enucleated animal cells. Drawn about 2 days after the operation. Note the strong animal influence displayed by larvae b, c and e.

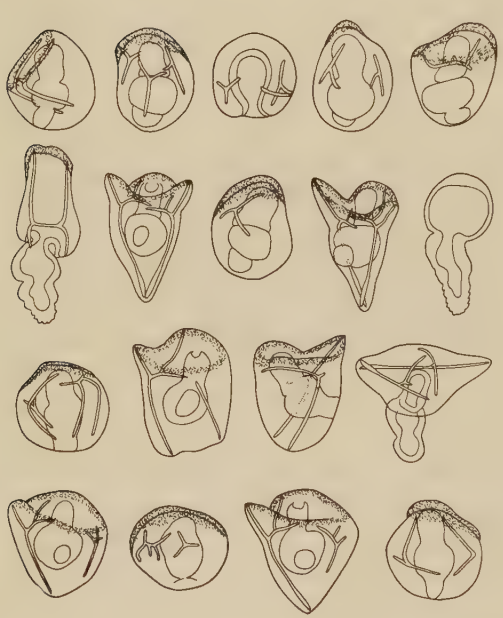

Fig. 4.

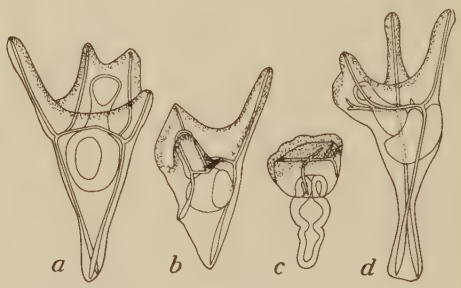

Fig. 5.

Figs. 4 and 5. Enucleation of the animal half. 1950 experiments. Second batch. (19/7 on Table III.) Fig. 4 shows the control vegetal halves. Fig. 5 a–d: vegetal halves with enucleated animal cells. Note the strong animal influence displayed by larvae a, b and d.

or rudimentary arms. The others are ovoid types and have no mouth. One of the 4 operated larvae is an exogastrula (Fig. 5 c) but the other 3 are plutei of more typical shape than any of the control larvae. Many of the arms are well developed.

The skeleton is formed by the most vegetal cells, i.e. the micromeres which form the primary mesenchyme, but the skeleton-forming cells cannot

produce a typical skeleton in the absence of a certain amount of animal properties. The ectoderm determines the pattern and induces the outgrowth of the skeletal rods (Lit. in 3). The irregular and poor development of the skeleton, the lack of or poor development of arms and ciliated bands, and the frequent absence of a mouth in the control larvae (Fig. 3 a_1–a_5 and Fig. 4, as well as the operated larvae as in Fig. 3 d and Fig. 5 c) indicate a deficit of the vegetal principle relative to the animal. In all the other larvae with enucleated animal halves the balance is nearly typical. It seems difficult to avoid the conclusion that the anucleate cells have exercised some strong animal activity on the adjoining vegetal half.

Before concluding that this demonstrates a strong influence of anucleate animal cytoplasm on its contiguous vegetal half, one must be absolutely certain that there is no possibility of any one of the presumably enucleated cells retaining its nucleus. The operated eggs were inspected after the cleavage leading to the 16-cell stage. All halves forming 4 micromeres were regarded as vegetal halves with enucleated animal blastomeres. They were again watched at intervals, as a rule in the 64-cell stage and later. From these observations it seems certain that the enucleation had succeeded in all cases, i.e. that the enucleated blastomeres did not divide normally nor take part in the development. There could be one possible exception, Fig. 5 d, in which case the larva was not observed between the 64-cell stage and the gastrula stage.

Gradual diminishing of the animal material in the 16- or 32-cell stages results in larvae intermediate in type between normal plutei and ovoid larvae, as a rule with enlarged digestive tract but reduced mouth ((2), p. 315). The plutei obtained in our experiments look more like normal plutei than one would expect if one animal blastomere had, by chance, not been properly enucleated and instead taken part in the development.

(II.) *1951 Experiments*

a) *Animal halves with enucleated vegetal cells.* 30 of the 31 halves judged as animal halves with enucleated vegetal cells developed in accordance with the controls. The exception was a larva which formed a very small archenteron and a triradiate spicule. There was also one more larva in which the intact blastomeres cleaved evenly, but this must in fact have been a meridional half with suppressed micromere formation. It was already considered as such at the late blastula stage, and it later developed into a pluteus. This larva is therefore omitted here.

Table II

Animal halves with enucleated vegetal halves. Table showing extent to which the surface of the blastulae formed from animal halves was covered by cilia (3/4 to 1/6), and the later differentiation (A–D).

	3/4	1/2	1/3	1/4	1/6	
Animal halves with anucleate veg. cells	4	7	12	7	1	= 31
Percentage	14	22	38	22	4	
Controls	15	12	22	23	4	= 76
Percentage	20	16	29	30	5	
	A	Ba	Bc	C	D	
Animal halves with anucleate veg. cells	6	7	6	1	1	= 21
Percentage	29	34	29	4	4	
Controls	12	14	14	12		= 52
Percentage	23	27	27	23		

A comparison of the larvae gives the following result (cf. Table II). The material comprises 6 series of experiments.

Considering the variety of types within the same batch of eggs and the rather small number of cases in each series, the deviations between operated cases and controls do not signify any differences between the two groups. The exception mentioned above is difficult to explain. The larva might be an animal half influenced by vegetal cytoplasm but more probably it contained an intact vegetal cell which failed to form a micromere and so remained undetected. It has a dwarf archenteron and skeletal spicules and resembles in type larvae obtained when, for instance, two micromeres are implanted into an animal half (4 implanted micromeres give plutei).

The results for 1950 and 1951 all show that the enucleated vegetal cells have not changed the differentiation of the animal halves. This result confirms the earlier experiments with anucleate cytoplasm fragments mentioned above (p. 264).

b) *Vegetal halves with enucleated animal cells.* Regarding the converse experiment, i.e. enucleation of the animal cells, the following results were obtained. The material comprises 29 halves from 6 batches of eggs, in which the operation was successfully performed, and it could be stated from fre-

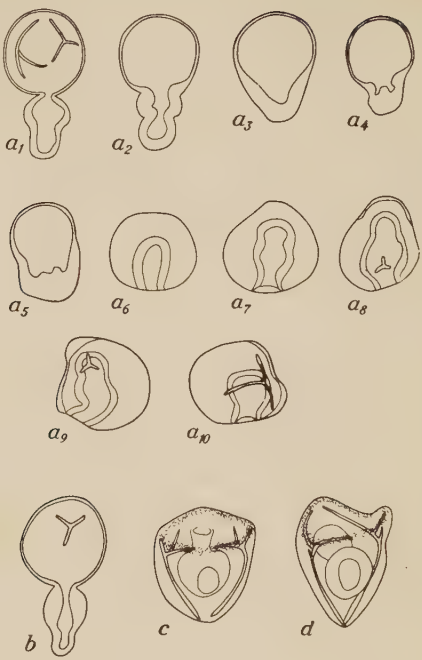

Fig. 6. Enucleation of the animal half. 1951 experiments. First batch. (23/6 on Table III.) a_1–a_{10}. The control vegetal halves. b, c and d. Vegetal halves with enucleated animal cells. Note the animal influence displayed by larvae c and d.

quent inspection that the enucleated cells did not divide regularly nor take part in the development. In one of these series, the 3 operated eggs became plutei but this does not reveal any influence on the part of the enucleate cells as all the control vegetal halves of this batch also formed plutei. In 4 series with 23 cases no deviation towards a more harmonic differentiation than that of the controls could be observed, thus giving a result in striking contrast to the 2 series from 1950. Only 1 series resembled those of 1950 (Fig. 6). Whereas the controls (a_1–a_{10}, fig. 6) were of a very "vegetal" type with 5 exogastrulae and the other 5 gastrulae with very poor development of the skeleton, the operated specimens gave 1 exogastrula (b, fig. 6) and two larvae of pluteus type (c and d, fig. 6) although with hardly any arms. Only one of them formed a mouth but both had a well developed skeleton as well as a ciliated band (which is missing in all control halves). These two larvae therefore clearly have differentiated in a much more "animal" manner than all the other halves of this batch. Thus, including the six 1950 plutei and the two 1951 plutei, we feel inclined to believe there is an influence of the enucleated animal blastomeres on the vegetal cells.

TABLE III

Summary of Results of Enucleation of Animal and Vegetal halves.

Batch no. (date)	Animal halves		Vegetal halves		Comment
	Developed	Influenced	Developed	Influenced	
1950					
$^{18}/_{7}$	3	0	4	3	
$^{19}/_{7}$	3	0	4	3	
1951					
$^{23}/_{6}$	3	0	3	2	[1] Probably not a pure animal half.
$^{6}/_{7}$	5	1[1]	3	0[2]	[2] 3 vegetal halves formed plutei but did not differ from controls.
$^{9}/_{7}$	6	0	3	0	
$^{12}/_{7}$	8	0	6	0	
$^{18}/_{7}$	3	0	5	0	
$^{26}/_{7}$	6	0	9	0	Some enucleated cells later removed (see text).
Total	37	1	37	8	

DISCUSSION

All the 6 animal halves observed in 1950 and 30 of the 31 cases in 1951 developed in accordance with the controls, i.e. they revealed no influence of the enucleated vegetal cytoplasm. As has been shown, the one exceptional case does not present any evidence for such an influence.

The results with vegetal halves contiguous with animal cytoplasm are more puzzling. In 1950, 6 of the 8 cases, and in 1951, 2 of the 29 cases, seem to show a marked influence in the animal direction (figs. 3–6). But it is remarkable that these results could not be repeated in the bulk of the 1951 results. The 2 positive 1951 cases belong to the first series (Fig. 6 and Table III); all the following 5 series were negative.

It was thought that the mass of enucleated cells, some of which may become enclosed in the blastula cavity, might hinder the development of the larvae by purely mechanical means or in some cases cause some damage when disintegrating. Perhaps more typical larvae might be obtained if the enucleated cells were removed after some time? Hence in the last series in 1951 the following experiment was done: in 3 of the 6 animal halves and in 3 of the 9 vegetal halves the anucleate cytoplasm was removed with a glass needle $3\frac{1}{2}$ to $4\frac{1}{2}$ hrs after the enucleation. But these halves did not develop differently

from the halves in which the enucleate cells remained outside or inside the developing blastula.

We have mentioned above the earlier negative experiments with implantation of most vegetal (micromere-forming) cytoplasm of a fertilized egg into animal halves. In our experiments with blastomeres enucleated at the 8 cell stage it is not the most *vegetal* but the most *equatorial* cytoplasm of the vegetal half which remains in close contact with the animal blastomeres. Would most vegetal cytoplasm have an effect if taken from micromeres? The possibility should be taken into consideration that the most vegetal cytoplasm is not yet active shortly after fertilization but would obtain inducing power in the course of cleavage as a result of progressive metabolic processes. We know that its power to form micromeres appears a certain time after fertilization (1). For this reason some preliminary experiments, in which the micromeres in some 16 cell stages were enucleated and the anucleate cells implanted into isolated animal halves, were done. Owing to experimental difficulties no conclusive results were as yet reached.

Runnström (8) considered the animal and vegetal properties to be present in the sea urchin egg as two gradients, and Lindahl (5) suggested metabolic differences between the two principles (for reviews see (6, 4)). As the two principles certainly differ qualitatively, it is not absurd that one of them could be influenced by anucleate cytoplasm but not the other. However, we do not venture to assert that this is the case. So far it has not been possible, either in earlier or in our present experiments, to prove any influence of anucleate vegetal cytoplasm on animal cells. On the other hand some of our results are difficult to explain except on the basis of an action of anucleate animal cytoplasm on vegetal cells.

SUMMARY

1. Sea urchin blastomeres at the 8 cell stage were enucleated in such a way that either the 4 animal or the 4 vegetal cells were deprived of their nuclei.

2. The enucleated cells usually formed asters and always divided irregularly, but they were never observed to differentiate or become incorporated into any larval organ or structure.

3. Of the 37 animal halves with enucleated vegetal cytoplasm which developed into larvae, 36 revealed no vegetal influence as compared with the control animal halves. The interpretation of one larva was inconclusive.

4. Of the 37 vegetal halves with enucleated animal cytoplasm, 8 revealed

a marked animal influence, i.e. they formed more or less well developed plutei whereas the control vegetal halves never displayed a comparable degree of differentiation.

5. Six of the 8 positive results with vegetal halves were obtained in 1950 and only 2 in 1951, although the number of experiments done in 1951 was 29 as compared with 8 in 1950.

6. The tentative conclusion is that enucleated vegetal cytoplasm does not influence the differentiation of the animal half, but enucleated animal cytoplasm may influence the vegetal half.

We should like to thank Professor G. Teissier, Professor P. Drach, Dr. Ch. Bocquet, Dr. C. Lévi and the staff of the Station Biologique, Roscoff, France, for their cooperation in obtaining the necessary material. Part of the expenses was defrayed by a grant from the Royal Society to Professor J. F. Danielli, and by a grant from the Swedish Research Council to Professor Hörstadius, for which we should like to express our thanks.

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ELEKTRONENMIKROSKOPISCHE BEOBACHTUNGEN ÜBER DIE ENTSTEHUNG DER MITOCHONDRIEN AUS MIKROSOMEN

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Die rein morphologische Forschungsrichtung wurde in den letzten Jahren immer mehr von einer experimentellen, nach funktionellen Zusammenhängen suchenden, abgelöst. Dies hat auch zu neuen Kenntnissen über Funktion und Bedeutung der Mitochondrien geführt.

Sie sind z. B. die Träger der zyklischen Dehydrierungsfermentsysteme (7) und damit die Erzeuger der zu den biologischen Synthesen so wichtigen energiereichen Phosphatbindungen (11). Sie wandeln also die in den Nährstoffen gespeicherte Energie in die im Stoffwechsel direkt verwertbare Form um.

Sie besitzen auch Speicherfunktion für verschiedenste Stoffe wie Hämoglobin, Saccharose, Heparin, Trypanblau etc. (zusammenfassende Darstellung siehe (16)).

Ein noch nicht gelöstes Problem ist jedoch die Frage nach der Entstehung der Mitochondrien. Die einen Autoren — vorwiegend Botaniker — vertreten die Ansicht, dass sich Mitochondrien nur aus ihresgleichen, d. h. aus den schon vorhandenen Mitochondrien, bilden, wie dies von den Plastiden gewisser Pflanzen, die ebenfalls nicht de novo entstehen, bekannt ist (6). Zytologen, die vorwiegend mit tierischem Material arbeiten, neigen dagegen mehr zur Annahme einer Mitochondrienneubildung aus kleineren Elementen.

Es wurden z. B. durch dosierte Röntgenbestrahlung des Pankreas der Maus die besonders empfindlichen Mitochondrien inaktiviert und deren Neubildung unabhängig von den geschädigten in der lebenden Zelle beobachtet (8).

Im tierischen Organismus kommt es somit sicher vor, dass die Mitochondrien nicht durch irgend einen Teilungsvorgang aus alten Mitochondrien, sondern aus neuen Elementen entstehen können. Welche aber sind diese Elemente?

Durch fraktionierte Zentrifugation von Leberhomogenaten wurde gezeigt, dass alle Grenzen zwischen den verschieden grossen Plasmagranula willkürlich sind (2); denn es lassen sich alle Übergangsformen sowohl in Bezug

auf die Grösse wie auch in Bezug auf den Fermentgehalt und die chemische Zusammensetzung isolieren.

Unter der Einwirkung der Edsall'schen Salzlösung konnten durch Zentrifugation von den Mitochondrien kleinere Granula abgegrenzt werden, die in Bezug auf den hohen Gehalt an Ribonukleinsäuren und ihre fermentativen Eigenschaften den Mikrosomen entsprechen (10). Der Bau der Mitochondrien wurde mit demjenigen von Viren (z. B. des Tabakmosaikvirus) verglichen, die z. T. ebenfalls aus verschiedenartigen, kleineren Elementen, die sich trennen und wieder vereinigen lassen, zusammengesetzt sind. Dabei sind für die Infektiosität, d. h. für die Selbstreproduktion in der Wirtszelle, diejenigen Elemente die wesentlichen, die sich durch ihren hohen Gehalt an Nukleinsäuren auszeichnen (10).

Auch im wachsenden Embryo findet man in den frühen Phasen reichlich kleinste Granula mit hohem Ribonukleinsäurengehalt (1). Mit zunehmender Differenzierung der Zellen geht die Zahl dieser kleinsten Elemente zu Gunsten der neu entstehenden Mitochondrien zurück.

Diese Tatsachen zeigen, dass bei der Entstehung der komplexen Elemente (Viren, Mitochondrien) die kleinsten, durch ihren Gehalt an Ribonukleinsäuren ausgezeichneten Granula (Mikrosomen) eine wesentliche Rolle spielen müssen.

Zollinger ist von einer neuen Seite an das Problem der Mitochondrienentstehung herangetreten (16). Bei Eiweisspeicherversuchen, die zu einem massiven Zerfall von Mitochondrien in den Epithelien der Nierenhauptstücke führen, hat er vermehrt kleinste Elemente im Zellplasma gefunden und sie als Vorstufen der Mitochondrien gedeutet.

Rüttiman ist darauf dem Problem der Speicherfähigkeit näher nachgegangen und hat gezeigt, dass dieselbe verschiedene Phasen durchläuft (14). Er injizierte Mäusen natives Hühnereiweiss intraperitoneal und stellte fest, dass dasselbe in den folgenden drei Tagen in zunehmendem Masse in den proximalen Hauptstücken der Niere unter dem Bild der hyalin-tropfigen Veränderung gespeichert wird. Die erst schlanken, dicht und basal gelagerten Mitochondrien wachsen dabei zu grossen, runden, die ganze Zelle anfüllenden Granula an. Wird täglich weiter Eiweiss injiziert, so tritt eine Erschöpfung des Mitochondrienapparates ein, und man findet nur noch spärlich kleine, mit Trichrom rot, nach Heidenhain schwarz gefärbte Granula und vereinzelt basale Stäbchen. Um wieder volle Speicherfähigkeit mit grossen Granula zu erhalten, ist ein injektionsfreies Intervall von 8 bis 10 Tagen nötig.

Schon Zollinger hatte im Phasenkontrastmikroskop zu Beginn dieses

Intervalles statt der grossen, hyalinen Granula vermehrt kleinste, erst mit Hilfe der Quellung durch dest. Wasser sichtbar zu machende Elemente feststellen können. Diese Ergebnisse deutet er in seiner Arbeitshypothese folgendermassen:

In der ersten Phase nehmen die Mitochondrien Eiweiss auf, bis sie gesättigt sind. In der zweiten Phase fehlen weitere speicherfähige, unverbrauchte Mitochondrien; es bilden sich aber zahlreiche kleinste Vorstufen (Mikrosomen), die in der dritten Phase zu Mitochondrien auswachsen und damit wieder speichern können.

Für uns stellte sich somit die Aufgabe, diese Hypothese der Regeneration der Mitochondrien aus kleineren Elementen, den Mikrosomen, mit objektiven Tatsachen zu belegen. Dabei müsste man bei der Neubildung von Mitochondrien eine kontinuierliche Reihe von Zwischenformen feststellen können. Da es sich aber um sehr kleine Partikel, an der Auflösungsgrenze des sichtbaren Lichtes handelt, lag es nahe, dies mit dem Elektronenmikroskop zu versuchen.

METHODIK

Die Anordnung des Tierversuches haben wir von Rüttimann übernommen (14). Ausgewachsenen weissen Mäusen wurde täglich 2 ccm steril entnommenes, natives Hühnereiweiss intraperitoneal injiziert. Wir untersuchten Tiere, die 1, 2, 5 und 6 Injektionen erhalten hatten. 24 Std. nach der letzten Injection wurden die Tiere durch Nackenschlag getötet, sofort durch die Carotiden ausgeblutet und seziert. Die lebensfrisch dekapsulierten Nieren homogenisierten wir in einem Zylinder mit eingeschliffenem, rasch rotierendem Glasstöpsel (13) unter Zusatz der 10-fachen Menge von 0,88 molarer Saccharoselösung, welche die Agglutination der Mitochondrien verhindert (9). Dann wurden die grösseren Bestandteile, wie Bindegewebsfasern, Erythrozyten, Kerne und Kerntrümmer während zwei Minuten bei 2000 g abzentrifugiert. Vom Überstehenden setzten wir je einen Tropfen auf ein mit einer ca. 100 Å dicken Kollodiumfolie bespanntes, auf Filtrierpapier liegendes Drahtnetzchen, wie sie als Objektträger für das Elektronenmikroskop gebraucht werden. (Zusammenfassende Darstellung der Technik siehe (3)). Wir liessen die Elemente im Tropfen 15 Minuten sedimentieren, brachten dann den Tropfen durch Kontakt mit dem Filtrierpapier zum Zerfliessen und spülten mit zwei Tropfen dest. Wasser sorgfältig nach, um die Saccharose zu entfernen und zugleich die Elemente künstlich zur Schwellung zu bringen. Die Drahtnetzchen wurden nun getrocknet und mit 40 mg Chrom unter einem Winkel von 22° in 20 cm Abstand vom verdampfenden Draht im Vakuum beschattet (15).

Um die Grösse der Teilchen leicht vergleichen zu können, machten wir alle Aufnahmen mit 10000-facher Vergrösserung und vergrösserten noch lichtoptisch auf das Doppelte.[1]

[1] Herrn Prof. Frey-Wyssling, Direktor des Pflanzenphysiolog. Institutes der ETH, Zürich, danken wir auch an dieser Stelle recht herzlich für die Erlaubnis, an seinem Elektronenmikroskop (Trüb-Täuber) arbeiten zu dürfen.

BEFUNDE

Wie schon andere gezeigt hatten (12), kann man auch im Elektronenmikroskop an typischen Mitochondrien einen kompakten Anteil, den Körper (Abb. 1. Mc), und eine dünne Membran (Abb. 1. Mb) erkennen, die sich durch die Einwirkung des dest. Wassers weit vom Körper abgehoben hatte. Diese Membran hat keine starre innere Struktur; d. h. ihre Form wird durch die Kräfte der Oberflächenspannung bestimmt. Wird sie lädiert, so bildet sie nie zackig begrenzte Bruchstücke, sondern zerfällt in kleine, flache Scheibchen (Abb. 1. Sch), die in allen Grössen vorkommen können. Es ist wichtig, dies zu beachten und diese Scheibchen von den korpuskulären Elementen (Mikrosomen, Abb. 1. Ms) gleichen Durchmessers zu unterscheiden. Dazu dient neben der grösseren Dichte der Letztern vor allem auch ihr anders geformter Schatten: der sichelförmige Schatten beim flachen Membranbruchstück hat praktisch überall die gleiche Breite, während beim korpuskulären Element entsprechend der zentral dicksten Partie auch die Schattensichel in der Mitte viel breiter als gegen ihre Enden ist (siehe Abb. 1).

Normale Vergleichstiere: In den Nierenhomogenaten von unbehandelten Vergleichstieren finden wir die Mitochondrien, wie sie oben beschrieben wurden, mit relativ schlankem oft leicht wurstförmig gebogenem Körper. Vereinzelt ist dieser auch etwas grösser und kugelig. Daneben finden sich immer zahlreiche kleinste, nur bis 0,1 μ grosse, korpuskuläre Elemente (Abb. 1. Ms), von denen sich keine Membran abgehoben hat. Sie entsprechen u. E. den als Mikrosomen bekannten Elementen. Zwischengrössen (Abb. 1. Zw) zwischen Mitochondrien und Mikrosomen, die z.T. auch Membranen besitzen, finden sich in Normalorganen nur ganz vereinzelt.

(Metallbeschattet, 20 000-fach vergrössert.)

Abb. 1. Suspension von *Normalnieren* mit schlanken, länglichen Mitochondrienkörpern (Mc_1), zahlreichen Mikrosomen (MS), die kleine halbmondförmige Schatten besitzen und vereinzelten kleinen, flachen Myelinscheibchen (Sch), die nur einen schmalen Schattensaum aufweisen. Daneben findet man eine Zwischenform (Zw).

Abb. 2. Nierensuspension *nach 1 Eiweissinjektion* vor 24 Stden (Speicherphase). Ein Mitochondrion mit grossem, rundem, kompaktem Körper (Mc_2), der Eiweiss gespeichert hat. Daneben ein Mikrosom (Ms) und einige Myelinscheibchen (Sch).

Abb. 3. Nierensuspension *nach 2 Eiweissinjektionen* im Abstand von 24 Stden, 24 Stden nach der 2. Injektion (Speicherphase). Zwei Mitochondrienkörper (Mc_3) umgeben von wolkig zerfallenden Eiweissmassen. Daneben zwei etwas verschieden grosse Mikrosomen (Ms) und drei Fremdkörperchen (Fk), erkennbar an der harten Begrenzung und dem kantigen Schatten. Das eine ist erst nach der Chrombeschattung auf die Objektträgerfolie gefallen und besitzt daher keinen Schatten.

Abb. 4. Nierensuspension *nach 5 Eiweissinjektionen* in täglichen Abständen (Erschöpfungsphase). Zahlreiche Zwischenformen (Zw) und einzelne nicht näher identifizierbare, fädige Strukturen (F).

Sch
Me1
Ms
Sch
Zw
Sch
Fig. 1
Zw
Zw
Zw
Fig. 4
Sch
Ms
Me2
Ms
Fig. 2
Fx
Me1
Fx
Ms
Fig. 3

1. Phase:

Speichertiere mit 1 Injektion zeigen selten mehr stäbchenförmige Mitochondrienkörper. Man findet statt dessen bis 5 μ grosse, geballte, für die Elektronen undurchdringliche Massen (daher keine Abb. davon) und kleinere, vorwiegend kompakte Kugeln ohne erkennbare Membran (Abb. 2. Mc). Daneben sind gleicherweise wie bei Normaltieren die Mikrosomen (Ms) vorhanden.

Nach *zwei Tagen Speicherung* hat sich das Bild insofern geändert, als die Mitochondrienkörper nicht mehr als kompakte Kugeln erscheinen, sondern eine wolkig zerfallende Struktur besitzen (Abb. 3. Mc).

2. Phase:

Nach *5 Tagen Speicherung* finden sich weder schlanke Normalmitochondrien noch die erwähnten grossen Speicherformen, sondern nur mittelgrosse, leicht zerfallende Kugeln. Daneben treten aber als wesentlich neue Erscheinung überaus zahlreiche, zwischen Mitochondrien und Mikrosomen einzuordnende Formen (Abb. 4. Zw) auf, wie man sie bei Normaltieren nur ganz vereinzelt findet. Sie besitzen einen zentralen dichteren Anteil und messen bis 0.5 μ. Eine eigentliche Membran scheinen sie nicht zu bilden.

3. Phase:

Bei Tieren, die an den ersten 5 Tagen je eine Injektion und dann erst am 10. Tag wieder eine solche erhalten haben, findet man *am 11. Tag* wieder die gleichen Bilder wie bei den Speichertieren nach 1 Injektion (wie Abb. 2), nämlich grosse, kugelige Mitochondrienkörper ohne Membran und daneben kleinste Mikrosomen. Die Zwischenformen fehlen wieder fast vollkommen.

DEUTUNG DER BEFUNDE

Quantitative chemische Analysen abzentrifugierter Plasmagranula haben ergeben, dass letztere, je kleiner sie sind, umso weniger Lipoide enthalten (2). Die Fähigkeit der Membranbildung im hypotonen Milieu nimmt ebenfalls mit der Teilchengrösse ab. Wir stellen uns daher vor, dass diese Membranen eine Lipoid-Eiweisstruktur besitzen, wie sie für die Myelinfiguren angenommen wird, in denen Schichten von orientierten Lipoiden mit Eiweisszwischenlagen, die Wasser aufnehmen können, abwechseln (4). Gleichartige Membranen finden sich auch um die Chlorophyllscheibchen

der Chloroplasten (5). Es werden Membranen sein, die durch Permeabilitätsveränderungen vermutlich den Stoffwechsel zwischen der Umgebung und den von ihnen umschlossenen Elementen regulieren.

Durch Eiweisspeicherung werden die erst schlanken Mitochondrien zu grossen Kugeln. Dass die Kugeln vom zweiten Tag an schollig zerfallen, dürfte der chemischen Umwandlung, der „Verdauung", des körperfremden Eiweisses entsprechen, wie sie an der veränderten Färbbarkeit im histologischen Schnitt zu erkennen ist (16).

Wohl weist die angewandte Methode für quantitative Bestimmungen grosse Fehlermöglichkeiten auf (z. B. das Abspülen mit dest. Wasser). Da aber bei den sechs untersuchten Speichertieren von Phase 1 und 3, sowie bei den vier untersuchten Normaltieren in keinem der angefertigten Präparate (je 25 pro Tier) in einem Blickfeld gleichzeitig mehrere Zwischenformen gefunden werden konnten, diese jedoch bei den zwei untersuchten Tieren der 2. Phase sogar eigentliche Gruppen bildeten, kann diese Feststellung wohl nicht als nur zufällig gedeutet werden. Leider gelang es noch nicht, brauchbare Schnitte herzustellen, an denen sich das quantitative Verhältnis der verschiedenen Teilchen in den verschiedenen Phasen in der Zelle selbst exakt auszählen liesse.

Welche Bedeutung haben nun die in der 2. Phase so zahlreich aufgetretenen Zwischenformen?

Man muss sich bewusst sein, dass durch unsere Versuchsanordnung ungewöhnliche Anforderungen an den Mitochondrienapparat gestellt werden. Die forcierte Eiweisspeicherung hat zu einem enormen Verbrauch und einer entsprechend intensiven Neubildung geführt. Unter physiologischen Bedingungen werden die Mitochondrien nicht annähernd in solchem Masse zu Grunde gehen, wie dies in vorliegendem Experiment der Fall ist. Das wird ein Grund sein, warum man normalerweise Zwischenformen nicht häufig findet.

Wären diese Zwischenformen nur tröpfchenförmige Plasmaeinschlüsse, ohne Zusammenhang mit der Mitochondrienneubildung, so müsste man sie doch wohl auch in der 1. und 3. Speicherphase finden können.

Dass 1) in der zweiten Phase von den kleinsten Mikrosomen an kontinuierlich alle Zwischengrössen so zahlreich gefunden werden, 2) in dieser Phase normale Mitochondrien und damit auch die Speicherfähigkeit fehlen, 3) in der anschliessenden dritten Phase statt der Zwischenformen nun wieder voll ausgewachsene, speicherfähige Mitochondrien zu finden sind, kann wohl nicht anders gedeutet werden, als dass die Zwischenformen Nachschubelemente zur Mitochondrienneubildung sind.

ZUSAMMENFASSUNG

Nach schwerer Eiweisspeicherung zeigen die Nierenhauptstückzellen eine deutliche Reduktion ihres Mitochondrienmaterials, welche erst nach ca. 10 Tagen wieder voll ausgeglichen ist (14). In den vorliegenden Versuchen konnte die Weiterentwicklung von Mikrosomen zu Mitochondrien in derartigen Mitochondrien-armen Zellen stufenweise verfolgt werden. Die Regeneration des Mitochondrienapparates nach Erschöpfung durch Speicherung scheint somit von den Mikrosomen auszugehen und in einem Ausreifen der einzelnen Elemente zu bestehen.

Besonders herzlichen Dank möchte ich Herrn Prof. Dr. Zollinger für seine unermüdlichen Mithilfe bei dieser Arbeit aussprechen.

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ULTRAVIOLET RADIATION EFFECTS ON GROWTH PROCESSES OF *BLEPHARISMA UNDULANS*

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In the increasing volume of literature dealing with the effects of ultraviolet radiations on animal cells and protozoa, the emphasis with few exceptions has been on fission delay or dose-dependent survival rates (13). Recently Kimball and Gaither (20) included studies on effects of ultraviolet radiations upon autogamy and macronuclear changes in *Paramecium aurelia*. Other recent work has focused primarily upon visible light reversal of ultraviolet radiation injury (photoreactivation) upon fission, autogamy or nuclear changes (3, 16, 20, 26, etc.). The studies in the present paper add data on the effects of ultraviolet light upon regeneration and relate these effects to changes in fission rate and photolysis. Some data on the effects of ultraviolet radiations on conjugation were also gathered.

Blepharisma undulans, a red-pigmented heterotrichous ciliate was selected for these studies because the trophic stage is comparatively large and sluggish (approximately 150 μ long by 50 μ wide), therefore it can be easily manipulated for free hand cutting. Furthermore extensive studies on regeneration after cutting have been presented (22, 23, 24), therefore a background is available for regeneration studies. Also under certain conditions an extremely large size can be induced by canabalistic feeding, therefore large individuals are available for comparison with small. Conjugation and mating type behavior have likewise been described for the species (8, 23, 24, 25). The presence of the red pigment which can be bleached out by exposure to white light (9, 24) lends itself to additional interesting variations in the condition of the experimental animal for studies with ultraviolet light. Cyst formation in this strain of *Blepharisma undulans* was not observed, but occurs in the species.

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METHODS

In the studies reported here the effects of ultraviolet light upon regeneration of the anterior third of the animal, including the peristomial field and the cytostome, but excluding if possible the macronucleus, were made. The cytostome of the animal was removed by a single slice across the end of the peristomial field. The animal was left in Brandwein's medium in a deep depression slide under a low power dissecting microscope during the operation. In controls the time required for regeneration of the peristomial area and its organelles was found to be approximately 6 hours, a time lapse in substantial agreement with previous determinations by both Moore (22) and Weisz (23). Regeneration in *Blepharisma* is independent of food in the medium and will occur even in sterile saline solutions. However the animals were placed in bacterized lettuce infusion after the operation and the uptake of bacteria on regeneration was used to mark the return of normal functioning of the cytostome.

Usually about 50 animals were cut, and 12 of those cut most accurately were selected for isolation. For irradiation studies a few extra cut animals were placed in the quartz irradiation cell and from the total 12 were isolated.

Progeny from a single isolation were used in experiments. Mass clonal cultures were grown at room temperature (about 20° C) in lettuce medium inoculated with *Pseudomonas ovalis* (7). The windowless underground laboratory remained within one degree of 20° C most of the time. Organisms to be used for regeneration experiments were taken from the test tubes about one week after the initial inoculation, and thus about one week prior to conjugation which usually occurred on or about the 13th or 14th day after inoculation. Should no additional bacteria be added to the test-tube medium, gradual starvation occurs with reduction in numbers and subsequent development of the large cannibals concurrently with dwarf forms that fall prey to the large. However when cannibal giants were desired for experiments, they were produced by first enlarging individuals by previous feeding with Chilomonas and Khawkinea before setting them upon individuals of their own kind. Giants so produced were more alike in size. Ultimately with passage of time all the animals in a culture become small, spindle shaped and bleached, the pigment occurring in localized purple patches in the anterior and posterior regions of the animals rather than the granular dispersed red pigment. Upon the addition of bacteria at any point in this sequence of diminution, the animals assume their normal form usually by three days. If no bacteria are added the animals ultimately die.

Isolations subsequent to cutting or to treatment with ultraviolet light or to both were made in each of the spots of a 12-spot Klein agglutination slide containing lettuce medium previously inoculated with bacteria. On a given slide the entire group of 12 isolations were individuals which had received the same treatment. In all experiments the agglutination slides were maintained in moist chambers at room temperature and protected against entry of visible light by several thicknesses of black cloth.

In early exploratory experiments a 40-watt Westinghouse Sterilamp was used as a source of radiations because of the ease of manipulation. The animals were

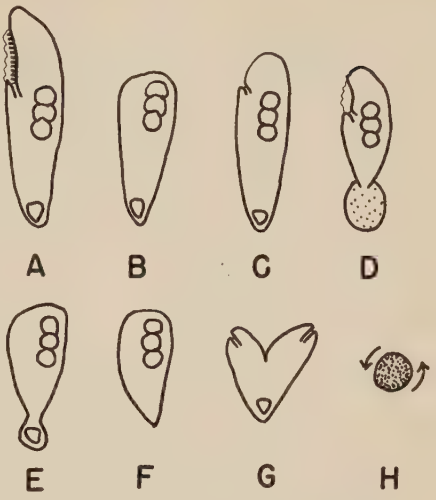

Fig. 1 A. Normal *Blepharisma undulans*. B. Unirradiated *Blepharisma* just after excision of anterior third of organism. C. Unirradiated regenerating form about one hour after excision. D. Unirradiated unexcised control showing abnormal fission. This appeared in a culture with an extremely dense suspension of bacteria. E. Cut individual exposed to injurious wavelength of ultraviolet light, showing onset of pinching off of posterior tip containing the contractile vacuole and surrounding area. (6 400 ergs/mm².) F. Same individual about one half hour later showing the form after completion of the pinching off of the area. G. Abnormal *Blepharisma* with two cytostomes produced by cutting and radiation exposure. (6 400 ergs/mm².) H. Rounded form produced by exposure of 6 400/mm² or more of ultraviolet light of injurious wavelengths.

exposed in deep depression slides and the dosage was determined with a Hanovia Ultraviolet Light Meter calibrated for Sterilamp radiations.

After the general features of the radiation responses and dosage ranges had been worked out with the Sterilamp, monochromatic ultraviolet radiations were used in order that the differences in action between different wavelengths might be studied. The animals were exposed in shallow depression fused-quartz slides or glass ringed quartz slides, since the ultraviolet radiations were passed through the slides from below. A monochromator with natural quartz optics (right and left hand quartz) was used and the intensity of the monochromatic light was measured by a thermopile previously calibrated against standard lamps. When pigment-free animals were desired, they were bleached at room temperature under a six watt daylight fluorescent lamp for 24 hours prior to use in cutting or irradiation.

In almost all cases a parallel set of uncut individuals obtained from the same clone were set up concurrently with a cut series and given the same dosage with ultraviolet light. Thus it was possible to get a comparison between effects of various kinds of treatment on cut and uncut individuals. Untreated controls otherwise under exactly the same conditions as the experimental animals were always maintained for all cut and uncut animals.

The main features in the regeneration of the peristomial field (primarily of the undulating membrane and mouth membranelles) are shown in Fig. 1, A, B, and C. In normal healthy animals the entire zone with its constituent organelles was usually reconstituted by about 6 hours. The organism then resumed active feeding and usually divided at least once in the first 24 hours after surgical treatment. The above regeneration pattern but with a delay was found to obtain after exposure to ultraviolet rays.

The number of animals in the entire series of experiments was not large because each one had to be handled and manipulated to a considerable degree and observations had to be made over a long period of time thereafter. Thus groups of 72 ani-

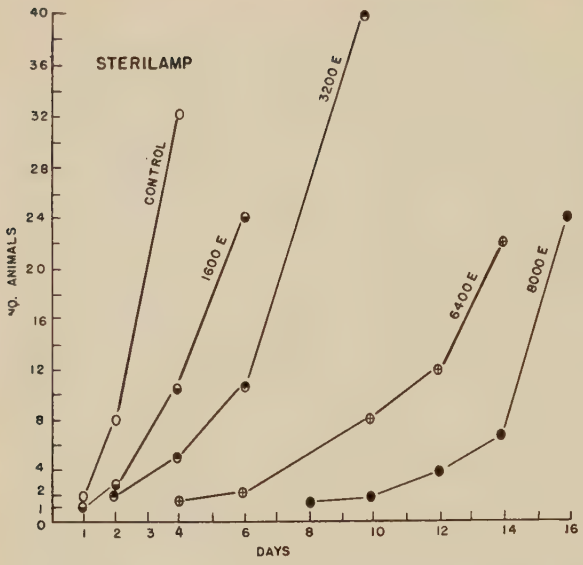

Fig. 2. Comparison of the effects of different dosages of sterilamp irradiation (mainly 254 mμ) on the fission rate of *Blepharisma undulans*. The "E" represents ergs/mm².

mals were used for each experimental series (e.g. 12 for control, 12 for a dosage of 3 200 ergs/mm², 12 for a dosage of 6 400 ergs/mm², cut and uncut in each case). In most cases two or three series of experiments were performed but only one series was carried out at each of the following wavelengths: 245, 302, 313 and 366 mμ. What was lost by lack of larger numbers was made up in the knowledge of the behavior of each individual in the course of all the treatments. The general uniformity of performance gives confidence in the data in spite of the small sampling possible by such methods.

Comparisons of effectiveness of different wavelengths was made on the basis of total incident energy. Since light acts in units of quanta, it might appear desirable to make such comparisons on the basis of quanta delivered per unit area rather than on the basis of total energy delivered per unit area. However if the quantum efficiency in producing an effect is not the same across the spectrum investigated, the comparison on the basis of incident quanta loses some of its meaning. Since the mechanism of action of ultraviolet radiations on cells is still so uncertain, it seems preferable to leave the data in terms of energy per unit area. By dividing each value by the energy in a quantum measured in ergs one can readily convert the energy values into numbers of quanta.

OBSERVATIONS

Effects of ultraviolet light from a Sterilamp. Dosages of the radiations from a Sterilamp (primarily) 254 mμ required to produce fission delay, regeneration delay and cytolysis were first determined. The effects of dosages of

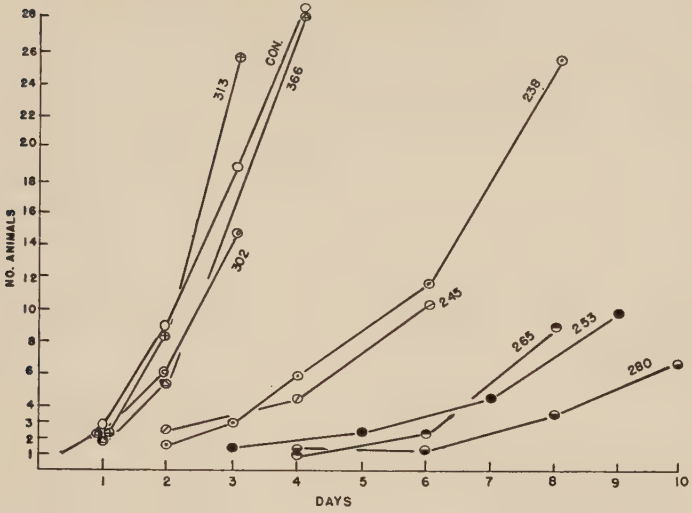

Fig. 3. Comparison of the effects of a dosage of 6400 ergs/mm² at various wavelengths of monochromatic ultraviolet light upon the fission rate of *Blepharisma undulans*.

800, 1 600, 3 200, 6 400 and 8 000 ergs per mm² upon the fission rate are presented in Fig. 2. It was found that little difference exists between the sensitivity to these radiations of the whole uncut *Blepharisma* and the cut animals. The graph indicates that the tolerance doses for *Blepharisma* are of the same order of magnitude but higher than those obtained by other investigators using protozoan material (13, 20, 21). After irradiation with large dosages the anterior and/or posterior ends of the exposed organisms may pinch off (see Fig. 1, E and F). Thus after a dosage of 6 400 ergs/mm² an organism was produced that lacked both the anterior peristomial fields and the posterior vacuolar area.

Studies of effects of monochromatic ultraviolet light upon fission delay. Since dosages of 3 200 and 6 400 ergs per mm² with the Sterilamp measurably retarded division rate of *Blepharisma*, the effects of such dosages with monochromatic light of various ultraviolet wavelengths was next studied. Fig. 3 represents the fission delay produced by a dose of 6 400 ergs/mm² at wavelengths: 238, 245, 254, 265, 280, 302, 313 and 366 mμ. The results can be classified into three groups: 1) the long wavelengths of ultraviolet light 302, 313, and 366 mμ which have little or no effect upon fission; 2) wavelengths 238 and 245 mμ which retard fission to some extent; and 3) wavelengths 254, 265 and 280 mμ which retard fission markedly.

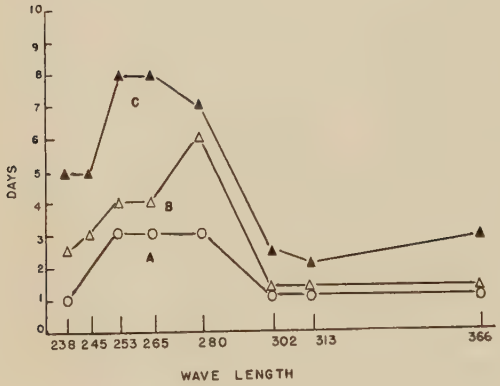

Fig. 4

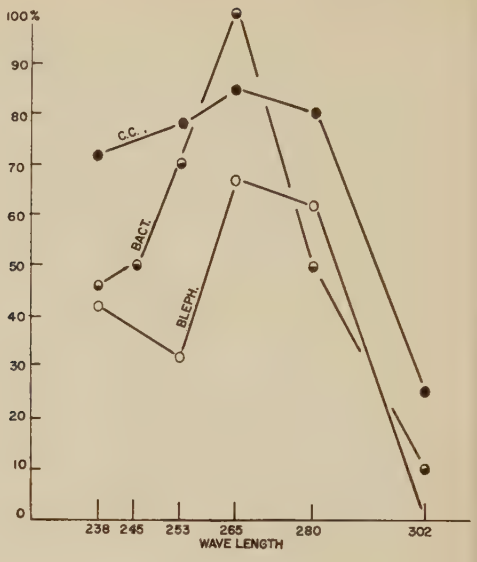

Fig. 5.

Fig. 4. Action spectrum of regeneration, first fission and third fission delays after dosages of 6400 ergs/mm² at the wavelengths indicated. A. Regeneration. B. First Fission. C. The third fission delay. The increased delay indicated at wavelength 360 mμ is probably an artefact.
Fig. 5. Action Spectrum of the killing effect of 6400 ergs/mm² compared to the bactericidal effect (2.) In addition, a composite curve for a mixture of proteins and nucleic acid is added (C.C.) The data for the latter were taken from Blum (2).

Action spectra for fission delay and regeneration: Fig. 4 A presents the action spectrum for the effect of a dosage of 6 400 ergs/mm² at various ultraviolet wavelengths on delay in regeneration; Fig. 4 B gives the delay in the first fission; and Fig. 4 C gives the delay of the third fission following a similar exposure. The data indicate that the wavelengths most effective in producing fission delay were also most effective in retarding regeneration. A dosage 3 200 ergs/mm² did not produce a readily measurable effect upon regeneration although the dose was sufficient to retard division. Regeneration is retarded most strongly after a dose of 6 400 ergs/mm² at λ 254, 265 and 280 mμ and similarly for all three wavelengths. The marked effect at 280 mμ upon the delay in the first fission is noteworthy compared to a lesser effect of the same wavelength on the third fission.

Survival and Photolysis. A dosage of 8 000 ergs/mm² with a Sterilamp causes cessation of ciliary locomotion in all groups of *Blepharisma* studied: whether bleached or unbleached, cut or uncut. The first blisters appear

after such a dose in all cases as well. Complete photolysis of the unbleached *Blepharisma* followed doses of about 16 000 to 20 000 ergs/mm² with the Sterilamp, as determined by twenty trials, whereas the bleached animals showed a greater sensitivity, a smaller dosage sufficing to cytolyze. A few experiments with cut bleached animals showed them to be as susceptible as uncut animals.

Fig. 5 presents the survival rates at several ultraviolet wavelengths after doses of 6 400 ergs/mm². Animals irradiated with wavelengths longer than 280 mμ did not die.

After irradiation with 238 mμ some individuals died within 24 hours; those which survived this period recovered completely, suggesting an all-or-none effect within the first 24 hours after exposure. Unfortunately observations were not made periodically and the work could not be repeated because the intensity at this wavelength of the arc declined to the vanishing point. Light of this wavelength produces qualitatively different effects from others observed. Specimens of *Blepharisma* exposed to radiations of this wavelength were immobilized and shrunken, the immediate shrinkage appearing superficially to resemble a dehydration effect. However those not killed by a dose of 6 400 ergs/mm² had recovered completely 24 hours later. Doses of 1 600 and 800 ergs/mm² only temporarily immobilized Blepharisma and did not affect the rate of fission.

Action spectrum of Survival. The action spectrum for killing *Blepharisma* after a dosage of 6 400 ergs/mm², an action spectrum for the bactericidal effect and a composite curve for absorption by a mixture of nucleic acid and protein (Blum 2)[1] are all given in Fig. 6. The data for killing of *Blepharisma* best fits the composite curve for a mixture of nucleic acid and protein. It is quite different from the bactericidal action spectrum which has a maximum at 260 mμ, thus matching well the absorption spectrum of nucleic acid.

Tests for Photoreactivation. The resistence of bleached and unbleached *Blepharisma* to ultraviolet light of λ 265 mμ as well as some data on photoreactivation are presented in Fig. 6. Similar results were also obtained using the Sterilamp but are not given in the graph. The graph is based upon data from four groups of animals isolated from a single clone. In each of the four groups with four individual isolations per group, one serves as control (C) while the other three are given different radiation treatments which are explained in the legend to the figure.

[1] We are indebted to Dr. Sylvia Frank of the Biology Department, Washington Square College, New York University, for assistance in interpretation of some of these data.

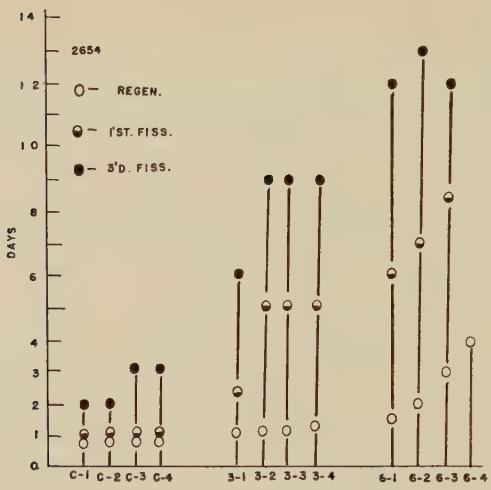

Fig. 6. Effects of 2654 A on bleached *Blepharisma* and attempts at photoreactivation. C–1: Controls for the unbleached group, receiving no radiation or illumination. C–2: Controls for the unbleached group, receiving no radiation, but one hour of visible light illumination. C–3: Controls for the bleached group receiving no radiation or illumination after bleaching. C–4: Controls for the bleached group receiving no radiation, but one hour of visible light illumination after bleaching. 3–1: Unbleached group receiving 3200 ergs/mm² with no subsequent illumination. 3–2: Unbleached group receiving 3200 ergs/mm² with one hour subsequent illumination. 3–3: Bleached group receiving 3200 ergs/mm² with no subsequent illumination. 3–4: Bleached group receiving 3200 ergs/mm² with one hour subsequent illumination. 6–1: Unbleached group receiving 6400 ergs/mm² with no subsequent illumination. 6–2: Unbleached group receiving 6400 ergs/mm² with one hour subsequent illumination. 6–3: Bleached group receiving 6400 ergs/mm² with no subsequent illumination. 6–4: Bleached group receiving 6400 ergs/mm² with one hour subsequent illumination.

From the graph it can be seen that the unbleached animals irradiated but not subsequently illuminated (the "1" series, i.e. 3–1, 6–1; C–1 being the control) recovered the most rapidly from both cutting and irradiation. When such unbleached animals were subjected to one hour illumination subsequent to irradiation (the "2" series, C–2, 3–2, 6–2; C–2 being the control) they recovered less rapidly. Bleached animals, used in the "3" series (3–3, 6–3; C–3 being the control), show about the same sensitivity as the unbleached. However, when bleached animals were irradiated then illuminated for an hour (the "4" series, C–4, 3–4, 6–4; C–4 being the control) no recovery from ultraviolet radiation was observed as a result of illumination and in some cases there was considerable damage from the one hour of visible light illumination. This is most clearly seen in the group receiving an ultraviolet dose of 6 400 ergs/mm² followed by an exposure to visible light (6–4); in which group none of the individuals reached even the first fission. Yet in all cases the controls illuminated for the same period

of time with visible light alone were in no way damaged. Thus there is no evidence of photoreactivation and evidence of an additive damaging effect of visible light something like heat after ultraviolet (17).

Effects upon Conjugation. Appearance of conjugants in cultures of irradiated organisms (and controls) used in the above experiments indicates that a dosage of 3 200 ergs/mm² does not prevent conjugation at any of the wavelengths tried. However, after a dosage of 6 400 ergs/mm² at λ 254, 265 and 280 mμ. (Conjugants appeared only in one group of 12 individuals which had been given this dosage at λ 280 mμ). The possibility that conjugation is blocked by such dosages of radiations merits further study.

Effects upon the Giant Cannibals. Since the linear dimensions of *Blepharisma* cannibals are usually about twice the normal, the possibility of testing the relations between dose of radiation and size of the organism was presented (21). Results with λ 253 mμ indicate that the giant form is as sensitive or possibly somewhat more sensitive than the normal-sized individuals. However, giant forms may be more sensitive than the controls because they are less pigmented.

It was found that organisms in early stages of fission with the cytoplasmic indentation becoming apparent when exposed to radiations at λ 254, 264 and 280 mμ exhibited the same degree of regeneration delay and fission retardation as non-dividing animals (see Figs. 3 and 4). Also forms in advanced fission with the cytoplasm markedly separated, but connected by a thin strand, divided within 24 hours after irradiation and the progeny of these forms exhibited the same fission delay for the next fission as non dividing animals.

Abnormal types of *Blepharisma* shown in Fig. 1 G were found at the first fission of cut animals treated with dosages of 6 400 ergs/mm² or 8 000 ergs/mm² at λ 254, 265, and 280 mμ. They were triangular-shaped with two cytostomes and incomplete peristomial fields, usually lacking the membranelles as well as the undulating membrane, and possessing one posterior area with a single contractile vacuole. Such individuals often persisted for approximately one week and then degenerated, becoming smaller and completely rounded with disappearance of all structure. In two instances each triangular abnormal form divided to give rise to a normal anterior zooid and a rounded degenerating posterior member. Sometimes cut irradiated animals gave rise to rounder abnormal individuals directly. The production of abnormal individuals may possibly be explained by the inadvertant damage of the macronucleus during cutting. However they were not observed amongst the controls. This conjecture is susceptible of proof by purposive damage to the macronucleus.

DISCUSSION

Ultraviolet radiation delays regeneration of *Blepharisma* from which the hypostome has been removed by cutting. The delay is most marked after treatment with λ 254, 264, 280 mμ. Since these wavelengths are absorbed strongly by nucleic acids, it is possible that the ultraviolet rays produce their effect by altering nucleic acid or nucleoproteins in the cell. Unfortunately the action spectrum for regeneration delay (Fig. 4 A) is too indefinite and lacking in detail to give support, although it is not in opposition to such a possibility. The weak action, upon regeneration, of λ 238 mμ which is strongly absorbed and produces striking superficial effects such as cessation of ciliary action, may lend some support to the argument. Light of this wavelength being superficially absorbed may not be able to reach the nucleic acids of the cell which are mainly in the nucleus and the surrounding areas (4). In the cytoplasm the nucleic acid may reside primarily in microsomes (5) and these may be important centers in protein synthesis (4). Protein synthesis is probably necessary for regeneration since the organelles which must be replaced are made up of proteins. The relation between nuclear action and regeneration may be profitably explored by suitable experiments using ultraviolet light.

After large dosage of ultraviolet radiations, *Blepharisma* often loses portions of its body—especially the tail end including the contractile vacuole, but sometimes also the anterior end. In that case recovery from these radiations includes regeneration much like that following cutting experiments. Such dropping of parts of a cell after ultraviolet radiation has not been previously described and its cause would be interesting to determine with more exactness.

The delay in division observed in *Blepharisma* after irradiation with ultraviolet light merely offers another example of this type of action of these radiations. The action spectrum for fission delay agrees with similar data for other cells such as eggs (13) and paramecia (11, 20). The delay in the first division of *Blepharisma* after irradiation is greatest for 280 mμ. By the time *Blepharisma* has undergone the third division, the delay for a dose at 280 mμ is less than at λ's 254 and 265 mμ. A similar phenomenon was observed with *Paramecium caudatum* in which recovery from large dosages of ultraviolet light was studied. The immediate effect on the early division subsequent to irradiation was obviously greatest at 280 mμ but recovery from this injury was rapid, whereas recovery from 265 was much slower. The order of efficiency of the two wavelengths therefore depended

upon whether measurements are made soon after irradiation or much later (11). This is suggestive of the qualitative difference in action of the two wavelengths.

Blepharisma seems to be more tolerant of ultraviolet radiations than most of the other protozoans tested and greater than some egg cells (13). This might be due to the presence of a red pigment (zoopurpurin, 1) an extract of which in alcohol is known to absorb strongly in the visible (6) and in the ultraviolet range (15). However the bleached *Blepharisma* while less resistant than the pigmented animals were also highly resistant—both as regards division and in resistance to cytolysis. Either the pigment is protective or the protoplasm of the organism is inherently more resistant for some other reason. Various protozoans show differences in resistance (10) as do marine eggs. No one has yet been able to place his finger on the factor which determines the resistance of a given type of cell to ultraviolet radiations.

The most striking feature of the present study is the failure to find any indications of photoreactivation. Thus when unbleached Blepharisma are irradiated with ultraviolet light and then exposed to visible light, they are retarded more than when subjected to ultraviolet light alone. The red pigment is known to be oxidized by visible light (18) leading to injury if the visible light is intense (12) or merely to retardation of division if the light is weak. However, the animals can be bleached until they appear colorless. Such animals might be expected to be less susceptible since they would absorb less visible light. Therefore colorless individuals were used in experiments parallel to the ones cited above. Those irradiated with ultraviolet light and then exposed to visible light were even more retarded in regeneration than those merely irradiated; furthermore the bleached animals which had been treated with ultraviolet and then with visible light were much more injured than unbleached animals similarly treated since they failed to divide even once, whereas the unbleached animals similarly treated did divide (Fig. 7).

It may be that we are dealing with two effects—a true photoreactivation of ultraviolet injury after treatment with visible light and an injurious effect of the visible light on the pigment (or on some product of the bleached pigment) and that the latter effect predominates. Even after bleaching, the residue of the pigment absorbs some of the light especially at the short end of the visible spectrum (15). Experiments excluding such portions of the spectrum are suggested as a possible way of differentiating between the two kinds of effects. All work on photoreactivation suggests that the short

end of the spectrum—blue, violet and long ultraviolet—are effective in reversing the action of short wavelength ultraviolet light (14, 16, 19, 26). Therefore separation of injurious from photoreactivating effects of visible light on *Blepharisma* is not possible.

SUMMARY

1. The effect of selected dosages of monochromatic ultraviolet light of wavelengths 238, 245, 253, 265, 280, 302, 313, 366 mμ were studied on the following: 1) regeneration of cut *Blepharisma*; 2) division rate; 3) conjugation; 4) survival of colored and bleached individuals.

2. No evidence for photoreactivation could be demonstrated in the ultraviolet injured animals.

3. Fission was most delayed at wavelength 280 mμ at the first division, but a somewhat longer delay was observed at 254 and 265 mμ for the third division.

4. The action spectrum for survival after large dosages of ultraviolet light best fits a curve that is a composite for a mixture of nucleic acid and protein, rather than either substance alone.

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A CYTOCHROME DEFICIENT MUTANT OF *SACCHAROMYCES CEREVISIAE*[1]

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This paper describes an ultraviolet induced cytochrome deficient mutant in yeast. Such a mutation is important since it permits the study of disruptions in oxidative metabolism, because yeast can live by fermentation alone without the absorption of oxygen.

The mutant was discovered in the following way. Lederberg (3) used the indicator 2,3,5 triphenyl tetrazolium chloride (TPTZ) to distinguish between strains of bacteria capable of fermenting various sugars and strains not capable of utilizing these sugars. In tests to determine if this technique could be used to distinguish fermenters from non-fermenters in yeast, cultures capable of fermenting sucrose produced red colonies on sucrose nutrient agar plates containing TPTZ, and cultures incapable of fermenting this sugar produced white colonies.

An attempt was then made to use this technique to isolate mutants unable to ferment glucose following ultraviolet irradiation. A few white colonies, which were thought to be non-fermenters, appeared among the several hundred red colonies which grew on the irradiated indicator plates containing glucose. However, clones derived from these white colonies fermented glucose as rapidly as those from the red colonies.

Instead of the "white colonies" differing from the normal "red-colonied" parent in the ability to utilize glucose, as was expected, the tests described herein showed that they differed from the normal in being unable to absorb oxygen. Furthermore, the inability to absorb oxygen is due to a deficiency in some of the components of the cytochrome system, and this deficiency is inherited as if it had resulted from the mutation of a single gene.

MATERIAL AND METHODS

The parent culture, which was irradiated to produce the mutant, is a haploid strain of *Saccharomyces cerevisiae*[2] derived from an extensive pedigree in which

[1] This work was begun at Southern Illinois University, Carbondale, Illinois, and continued at the Detroit Institute of Cancer Research, Detroit, Michigan, where it was supported by a grant from The Elsa U. Pardee Foundation.

[2] Culture number 8456 in the Lindegren collection.

various genetic factors have been introduced by crosses with a number of strains of *S. cerevisiae* as well as with other species of *Saccharomyces*. It is characterized as being able to ferment sucrose, maltose, melibiose, and galactose, and as being unable to grow without added biotin and pyridoxine.

A suspension of this culture was plated on three glucose nutrient agar plates containing 0.005 per cent TPTZ. The plates were irradiated for two minutes with ultraviolet irradiation, using a Hanovia ultraviolet lamp. They were light reactivated by placing the plates in sunlight for ten minutes immediately following irradiation and then incubated at 30° C until the appearance of colonies. From the several white colonies which appeared on the plates, one was selected for further study.

As it was not possible to begin work on the mutant immediately, it was stored in the refrigerator for several months. At the end of this time, when it was replated on a TPTZ indicator plate, a mixture of red and white colonies appeared. A single white colony (culture W-1) was isolated and used for all tests reported in this paper.

To determine the genetic nature of the variant, it was crossed with other cultures. Matings were made by inoculating a broth tube (medium No. 1) with a large loopful of yeast of each of the two opposite mating types scraped off stock slants (4). After incubating at 30° C for a day, an aliquot of the mating mixture was diluted and plated out on TPTZ indicator plates. Colonies believed to be diploid because they were larger and a different color than the haploids were checked for ability to mate with both mating types. If no copulations were observed with either mating-type test strain, the diploid isolates were transferred first to a presporulation slant and then to a gypsum slant to induce the formation of ascospores. The asci were dissected and spores isolated with the aid of a de Fonbrune micromanipulator.

Cultures grown from the isolated ascospores were tested to determine their mating type, their ability to absorb oxygen in an atmosphere of air and the presence of the typical cytochrome absorption bands.

Two essentially similar nutrient media were used in these experiments. Medium No. 1 contained the following, per liter: 6 g bacto yeast extract (or 5 ml Anheuser Busch liquid yeast extract), 3.5 g peptone, 2 g KH_2PO_4, 40 g cerelose, and 30 g agar, for solid medium. Medium No. 2 contained, per liter: 2 g $(NH_4)_2PO_4$, 3.5 g peptone, 2.5 g basamin bact, 40 g glucose, 2 mg each of thiamin, pyridoxine, and pantothenic acid, 0.5 mg biotin, and 0.1 mg each of p-aminobenzoic acid, nicotinic acid, inositol, and riboflavin. A third medium, Wickerham's yeast nitrogen base (Difco) was used with the addition of 4 per cent glucose and 3 per cent bacto agar (Difco) as a synthetic medium.

The parents and the haploid segregants of the various crosses were observed spectroscopically to determine the presence or absence of the cytochrome absorption bands. Yeast used for spectroscopic examination was grown on medium No. 1 in Petri dishes at 30° C. After from one to three days' growth the yeast was harvested by washing it from the agar plates with distilled water, centrifuging, and filtering through a Büchner funnel with suction. The yeast cake so formed was pressed between two glass microscope slides to a thickness of 2 mm and examined for absorption bands with a Zeiss direct vision hand spectroscope. In attempts to clarify the bands the cake yeast was either treated with sodium hyposulfite or cooled with liquid nitrogen. However, reduction with sodium hyposulfite produced no visible increase in the intensity of the bands in either the normal or the mutant. Cooling

culture W-1 with liquid nitrogen resulted in a somewhat sharper band at 550 mμ, but no new bands appeared. Since spectra of the various types of yeast were readily distinguishable in freshly harvested cake yeast, routine examinations of the cultures were made with fresh cake yeast.

Uniformity of the cultures classified as to cytochrome deficiency by observing the absorption bands was checked by plating on TPTZ agar plates. When the correlation between absence of bands and formation of white colonies had been adequately established, some of the progeny were classified on the basis of the TPTZ plates alone.

Respiration and fermentation tests on parents and segregants were carried out in the Warburg apparatus. Yeast suspensions were made up in distilled water to a density of 15 per cent transmission in the Coleman spectrophotometer; this is a concentration of about 1.7 mg dry yeast per ml. Aliquots of the suspensions used were dried to constant weight and the dry weight of the yeast determined.

The progeny of all of the crosses were tested for mating type by determining the ability to fuse with cells of both mating types when inoculated into broth tubes. In all cases cells of any spore clone fused with cells of one mating-type tested and not with the other.

RESULTS

When yeast grown on Petri dishes was tested spectroscopically, culture W-1 showed a single very faint absorption band at 550 mμ. Under the same conditions, the parent culture, 8456, exhibited the usual alpha bands characteristic of cytochromes-a, -b, and -c, at 605 mμ, 565 mμ, and 550 mμ respectively, as well as beta bands of cytochrome-b at 520 mμ and of cytochrome-c at 530 mμ. On the basis of the spectroscopic examination of yeast grown in standing culture, strain W-1 was originally described as exhibiting no cytochrome absorption bands (5).

The results of various experiments on oxygen absorption are given in Table I. It is apparent from Table I that while the parent 8456 is capable of taking up oxygen, the variant W-1 is not, whether grown aerobically or anaerobically.

In the routine check for variation in which the cultures derived from the haploid spore segregants were plated on TPTZ indicator plates, a great many small white colonies were observed among the large pink to red colonies of normal cultures. Spectroscopic examination of cultures derived from these small colonies revealed a strong absorption band at 550 mμ and a faint band at 520 mμ, the wavelengths at which cytochrome-c absorbs light.[1]

[1] Dr. Britton Chance reports that when the absorption of yeast cells of this type (culture 54.2-P, Table III) is measured spectrophotometrically it exhibits visible bands at 550 mμ and 520 mμ, and the Soret band at 424 mμ. He states that while the positions of the bands are similar to those of cytochrome-c, their intensities are dissimilar. (Personal communication)

TABLE I

Respiration by Normal and Cytochrome-deficient Yeasts.[1]

Expt.	Culture	Q O_2	Q CO_2	Q N_2/CO_2	Conditions of Growth
I	8 456	63	237	208	Standing, 250 ml medium No. 2 per 2 liter flask.
	W-1	−8	122	143	
II	8 456	55	267		Same as Expt. I.
	W-1	3	108		
III	8 456-P	3	285		Grown on medium No. 1 in Petri dishes.
	W-1	0	317		
IV	8 456	55	144		Same as Expt. III.
	8 456-P	0	266		
	W-1	0	299		

Thus, they were like culture W-1 and its progeny in the absence of the bands characteristic of cytochromes-a and -b but differed from them in the intensity of the absorption band at 550 mμ. The beta band at 520 mμ is correspondingly less intense in the mutant cultures, so that it is only rarely discernible. When tested on the Warburg, the spontaneous variants (cultures labeled 8456-P in Table I) took up no oxygen. These variants, therefore, have the appearance of the non-genetic "petites" described by Ephrussi *et al.* (1).[2]

In these strains, three types of cultures shown in Table I can be distinguished by the kind of colonies they produce on TPTZ agar plates. Culture W-1 and the mutant progeny derived from W-1 produce flat, small white colonies, which, after several days, gradually become pale pink. The apparently non-genetic petites form small white colonies that gradually develop a distinct dark red center. The wild type forms large colonies that are fairly evenly colored deep pink to red. As with the other types of colonies, the color of the wild type colonies becomes more intense as the colonies become older over a period of a week or two. The intensity also differs between

[1] Each flask contained 1 ml M/8 KH_2PO_4, 1 ml yeast suspension containing about 1.7 mg dry yeast per ml, 0.5 ml M/2 glucose, and 0.2 ml 15 per cent KOH or 0.2 ml water in the center well. Temperature 30° C. Atmosphere air or 99.7 per cent nitrogen.

[2] The indicator was apparently without effect on the growth of cytochrome deficient cultures but inhibited the growth of normal cultures. Only about 3 per cent of the normal cells that produced colonies on nutrient agar plates grew on plates to which 0.005 per cent TPTZ had been added.

TABLE II

Segregation of Cytochrome Deficiency in Crosses of Genetic by Non-genetic Deficients.

No. asci	Segregations
	25.1 × 25.2
4	C[1] C c c
4	C – c c
2	C C c –
	25.1 × 25.4
1	C C c c
1	C – c c
2	C C c –

various wild type cultures and apparently is affected by the segregation of other factors which may possibly alter oxidation-reduction reactions in the cells.

A cross was made between culture 8456 and a mutant clone descended from W-1, number 23.3, which was one of two mutant spores in a regularly segregating 4-spored ascus. Diploid III isolated from this cross yielded an unusual ascus, in which one spore clone (25.3) was normal, one was phenotypically like the apparently non-genetic petite (25.1), and two were mutant like W-1 (25.2 and 25.4). Crosses were made between the apparently non-genetic petite and the two mutant clones, both of which happened to be of the opposite mating type. The results obtained are given in Table II.

Eleven of the asci listed in Table II were dissected from the mating mixture and three from isolated diploids. The isolated diploids from both 25.1 × 25.2 and from 25.1 × 25.4 crosses were normal and contained all the cytochrome bands. All of the diploids tested produced some petite colonies when plated on indicator plates.

Spore germination is very low in these cytochrome deficient strains. The fourteen 3- and 4-spored asci listed in Table II were obtained from the dissection of a hundred or more 4-spored asci. In most cases, none or only one or two of the spores grew.

As shown in Table II, normal and mutant spores were found in equal numbers in the progeny of the petite by mutant crosses, in accordance with the 2:2 segregation expected in each ascus of a cross in which a single pair

[1] C indicates the wild type; c cultures are identical phenotypically with W-1 in that their spectra have a faint absorption band at 550 mμ.

TABLE III

Absorption Spectra and Respiratory Activity of Normal, and Genetic and Non-genetic Cytochrome Deficient Strains of Yeast.

Expt.	Culture	Alpha Bands	Q O_2	Q CO_2	Q N_2/CO_2
I	5.2-c[1]		<1	174	
	5.2-C		20	79	
II	54.2-c		2	185	212
	54.2-C		47	97	99
	54.2-P		1	210	219
III	5.2-c	550 mμ (faint)	1	235	226
	5.2-C	605, 565, 550 mμ	52	90	86
	54.2-c	550 mμ (faint)	0	206	217
	54.2-C	605, 565, 550 mμ	35	91	99
	54.2-P	550 mμ (intense)	10[2]	235	224
IV	54.2-c		0	249	
	54.2-C		82	169	
	54.2-P[3]		3	225	

of alleles segregates. The genes controlling mating type and the fermentation of melibiose and galactose which were used as markers in the cross, also segregated regularly. The crosses, therefore, confirm preliminary evidence (5) indicating that the ultraviolet treatment induced a gene mutation resulting in the cytochrome deficiency. No petites were recovered in the progeny, except that when each of the normal cultures was plated out on indicator plates a variable number of petite colonies appeared among the normal colonies. The petite character of spore clone 25.1, therefore, was not transmitted through the cross.

After a variable number of weeks' storage in the refrigerator, vigorous colonial outgrowths were observed on the stock slants in eleven of twenty mutant cultures derived from the cross of cultures 25.1 × 25.2. Most of these reverting cultures are listed in the mutant segregants shown in Table II giving asci in which three or four spores germinated. The others are from asci in

[1] 5.2-c and 54.2-c are the original mutant segregants; 5.2-C and 54.2-C are normal cultures derived from large outgrowths appearing on the stock slants; 54.2–P is derived from a petite colony which appeared spontaneously in culture 54.2-C.

[2] The reason for this petite culture taking up a small amount of oxygen is not known. In other tests of petite clones, little or no oxygen was absorbed.

[3] No normal colonies were produced on the aliquot plated on either TPTZ or plain agar, even among the several hundred mutant colonies which appeared on the plain agar plates.

which only two spores germinated and are not included in Table II. No such outgrowths were observed among the six mutant segregants of the 25.1 × 25.4 cross. Such vigorously growing colonies from two cultures (5.2-C and 54.2-C) are included in Table III. The parent culture 54.2-c is a mutant segregant from the cross 25.1 × 25.2, and culture 5.2-c is a mutant segregant from a previous cross between a normal and a mutant.

When the normal cultures derived from the apparent backmutants (5.2-C and 54.2-C) were plated out on TPTZ agar plates, a large number of petite colonies appeared on the plates. One of these (54.2-P) was tested and is included in Table III. Such petite cultures were stable and did not revert to the wild type throughout vegetative division.

In ten out of the eleven cultures derived from large-colonied reversions that were observed spectroscopically, absorption bands characteristic of cytochromes-a, -b, and -c were present. In the remaining culture derived from a vigorous outgrowth, only the faint 550 mμ band characteristic of the mutant segregant was visible.

In order to determine whether the reversion to normal was a back-mutation of the recessive gene or whether it was a mutation at another locus, culture 54.2-C was crossed with a normal culture. The diploid isolates obtained from this cross were normal, and the twenty haploid segregants isolated from the cross were all normal. Since no cytochrome deficient segregants were obtained, the outgrowths appear to be caused by back-mutation of the cytochrome deficient mutant gene to the normal.

DISCUSSION

The frequent occurrence of variants from the normal type that have a strong absorption band at 550 mμ, such as those described by Ephrussi *et al.* (1) as apparently non-genetic petite colonies, has been confirmed. On the basis of similar evidence, Ephrussi (1, 2) has postulated that the petites are due to loss of cytoplasmic particles necessary for the development of the complete cytochrome system. The recovery of normal diploids from a cross of two cytochrome-deficient haploids, petite by mutant, is consistent with this explanation. The recovery of a normal diploid can be explained by assuming first that the mutant parent contains self-reproducing cytoplasmic particles necessary for the development of the complete cytochrome system but lacks one of the necessary genes, and second, that the petite is genetically complete but lacks the necessary cytoplasmic constituent.

The regular 2:2 segregation of mutant to normal in all of the asci tested

indicates that the ultraviolet irradiation induced a gene change. The many examples of gene mutations resulting in step-wise blocks in the enzymatic synthesis of various biological compounds make it reasonable to infer that the mutation results in a block in the pathway of synthesis of the cytochromes. The presence of the faint absorption bands at 550 mμ and at 520 mμ indicates the presence of a small amount of cytochrome-c or a related compound with absorption maxima at the same wavelengths. The question as to whether the absorption bands are due to cytochrome-c or a slightly different compound is raised by the dissilimarities in intensities of the absorption bands of the petite cultures and cytochrome-c, as measured by Dr. Chance.[1]

The occurrence of a small amount of this cytochrome-c-like compound in the mutants could be accounted for either by a "leaky" gene or by its synthesis from a trace of some precursor present in the nutrient medium on which the yeast is grown. However, the faint band at 550 mμ is visible in mutant yeast grown in Petri dishes containing Wickerham's synthetic yeast nitrogen base medium (Difco) to which 4 per cent glucose and 3 per cent unwashed Difco bacto agar is added. If the band is due to a trace of precursor, the precursor must be present in this medium as well.

In any event, one would expect that a "leaky" gene or a trace of precursor in the nutrient medium would produce a faint but *complete* spectrum. That a trace of all the cytochromes is not produced might be accounted for by assuming that the mutants have lost the postulated cytoplasmic factor necessary for the production of the complete cytochrome system. However, this explanation is incompatible with the fact that although cultures 25.2 and 25.4 exhibit faint bands only at 550 mμ when grown on nutrient medium they produce normal diploids when crossed with petite culture 25.1. The cytoplasmic factor must be present in cultures 25.2 and 25.4 if normal diploids are reconstituted in crosses with petite cultures. Lack of the complete cytochrome system then cannot be accounted for by assuming loss of the cytoplasmic factor in these cultures.

It is possible that the compound exhibiting the absorption bands of cytochrome-c is formed preferentially. That is, when only a trace of precursor is present this compound may be formed rather than the entire cytochrome system.

The irregular occurrence of reversions of the mutants to normals is curious. Although a large number of reversions were observed on some of the stock slants, none were observed on other slants. One explanation for the lack of reversions in some clones may be that the mutant clones that did not

[1] See Footnote 1, p. 297.

revert had lost the cytoplasmic factor, and hence would be unable to reconstitute a normal cytochrome system even if mutation occurred. Support for this hypothesis comes from the fact that the ability to revert was lost on transfer on several occasions. For example, no reversions have been observed in culture W-1 since it was isolated, although reversions apparently had occurred prior to the time of isolation.

An attempt to demonstrate a difference between the presence of a cytoplasmic factor in reverting and non-reverting mutant strains by their ability to form normal diploids when crossed with petites was unsuccessful. There may be differences in the selective advantage of the apparent back-mutants among the various strains. That is, in some strains the large mutant population may have prevented the normal backmutant from growing, while in others the mutant population did not.

There is an indication that in some strains the rate of reversion to the normal is very high. When ascospores are dissected, each spore is placed in one of four marked-off squares on the surface of a single drop of nutrient agar on a cover slip, and incubated for 48 to 72 hours, by which time, each spore has produced a small "spore colony". A portion of this colony or the entire colony is then transferred under a dissecting microscope to an agar slant, which is used as a stock culture to furnish inocula for the various tests. The normal segregants are readily distinguishable from the cytochrome deficients in the spore colony stage. The normal spore produces a large colony that has a yellowish tinge, and the cytochrome deficient spore produces a very small colony that is pure white.

In a cross between a mutant and a normal culture, several 4-spored asci were obtained in which there were two large normal appearing spore colonies and two small white spore colonies produced by the four spores isolated. However, a few days after the small spore colonies had been transferred to stock slants large colonies appeared all over the surface of the growth on the slants, and in a short time completely overgrew the slants. When inocula were taken from the slants and grown in sufficient quantity to test for cytochrome, all four spore clones from each of the asci were normal. At the same time, the marker genes for mating type, the ability to grow without added thiamin, and the ability to ferment melibiose segregated 2 : 2, indicating that the segregation in this cross was perfectly regular. Apparently the rate of reversion was so high in the segregants from these particular diploids that the population reverted before it could be grown in sufficient quantity to test. This suggests that the stability of the mutant gene may vary in different diploids.

In one haploid mutant segregant of the cross 25.1 × 25.2 a reverting type of colony has been isolated which, after three serial isolations, continues to produce both normal and mutant colonies in the ratio of 1 normal to 5 ±1.9 reverting to 16 ±8.7 mutant. This dissociation is now under investigation. However, it is clear that the mutant is unstable and that reversions occur in some strains with a high frequency.

Since the tests commonly used to distinguish between normal and cytochrome deficient cells (e.g. presence of absorption bands, Nadi reagent, alcohol utilization, or oxygen uptake) require a mass of cells, any instability in the cytochrome character requires special consideration. Because of the frequent reversion of the mutant to normal and the high rate of transformation of normal to petite, the population grown from a mutant spore might be characterized as mutant, normal, or petite, depending on which variant had been selected during growth. Culture 25.1 may have been obtained in this way, the spore possibly having been normal, but a petite variant of this culture having been picked by chance for testing. A segregant cannot safely be characterized on the basis of the properties of a large clonal population of cells derived from it unless the variation among members of that clone has been determined.

SUMMARY

1. A mutant yeast which is incapable of absorbing oxygen was obtained by ultraviolet irradiation. The mutant does not exhibit absorption bands for cytochromes-a and -b and shows a marked reduction in the bands characteristic of cytochrome-c.

2. When this cytochrome-deficient mutant is crossed with petite, another cytochrome-deficient haploid, normal diploids are obtained. The appearance of normal and mutant yeast in a 1:1 ratio indicates that the cytochrome deficiency can be referred to the segregation of a single gene.

3. None of the haploid segregants are petite, but all the clones from normal spores produce some petite colonies when plated. These results can be accounted for if the petites are genetically normal but deficient in a cytoplasmic factor which is also necessary for the production of the complete cytochrome system.

4. A great many reversions to normal were observed among the clones from some mutant segregants. No reversions were found in other mutant segregants. These observations makes questionable the classification of normal and cytochrome deficient segregants by tests that utilize large masses of cells, unless the homogeneity of the population is verified.

I would like to thank Mrs. Carl C. Lindegren for supplying culture 8456, as well as other cultures used in various crosses, and also for making the first crosses which indicated that the change was genetic.

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STUDIES ON ISOLATED CELL COMPONENTS

V. THE EFFECTS OF VARIOUS SOLUTIONS ON THE NUCLEAR ENVELOPE OF THE ISOLATED RAT LIVER NUCLEUS

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The existence of a distinct nuclear membrane is no longer in serious doubt (10, 18, 21, 13, 15, 8). A number of observations have suggested that this structure is composed of at least two layers. Thus the nuclear envelopes of several amphibian oöcytes have been shown to consist of a perforated inner sheet or layer and a thin, apparently structureless outer layer (8). In isolated plant nuclei, blisters or blebs have been observed to rise from the main body of the nucleus (11, 14), while similar observations have been recorded for rat liver nuclei (3). Blebs have also been observed in nuclei of cells after treatment with neutral formalin (12). From birefringence studies it has been concluded that the envelope generally contains protein chains or plates running in a tangential direction (19) and also a variable amount of lipid (20). From similar studies Monné has concluded that the nuclear envelope is double with a firm nuclear protein layer free of lipids, and a very delicate cytoplasmic protein-lipid layer (18). Baud has also presented evidence for a perinuclear lipidic layer in the liver cell (6) based on histochemical techniques. These results, as well as those obtained by microdissection methods (9), demonstrate that the nuclear envelope is a structural entity and not merely a phase boundary. In view of the complexity of this structure and its evident lack of many of the properties which have come to be associated with the cell membrane (10, 16, 17, 7), it has been considered advisable in the present work to use the term "nuclear envelope" in place of nuclear membrane.

The nuclear envelope has been shown to be permeable to salts (1, 5, 8, 16, 17, 7) and to many low molecular weight substances (7). It appears to offer little resistance to the entrance of heparin or to the extrusion of

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highly polymerized desoxyribonucleic acid (DNA) (4). A number of proteins have been shown to penetrate it rather readily (2),

In the present study the results of observations of the effects of various solutions on the envelope of isolated rat liver nuclei will be presented. A number of factors influencing the formation of nuclear blebs or blisters have been investigated. As will be shown, the isolated nucleus possesses an envelope with properties not dissimilar from those previously described for nuclei of intact cells. The results obtained are discussed from the dual point of view of their possible application to nuclear physiology and to the evaluation of techniques for the isolation of nuclei.

EXPERIMENTAL

Methods. Rat liver nuclei were isolated in a phosphate-bicarbonate buffer containing sucrose[1] as previously described (22). Observations and photographs were made at room temperature (24–31° C) with the phase contrast microscope (5). All solutions were made up in double distilled water.

Observations on the effects of salt solutions. In a number of solutions, blebs or bubbles have been observed to appear on the surface of isolated nuclei. The blebs are generally rather small except in dilute solutions of $CaCl_2$ and $MgCl_2$ where blebs were often as large as the nuclei. Blebs which occur in 0.001 M $MgCl_2$ are shown in Fig. 1. It should be noted that blebs in contact with each other do not coalesce in a manner characteristic of soap bubbles, but remain discrete.

The effect of a graded series of concentrations of KCl, NaCl, K-phosphate (pH 7.0–7.4), $MgCl_2$, and $CaCl_2$ on the formation of blebs by isolated rat liver nuclei is shown in Table I. In many of the solutions the blebs were numerous but small (ca. 1–3 μ). This was especially true of dilute NaCl, KCl, K-phosphate solutions, and also distilled water. It should be noted that the figures given in Table I are minimal, since only blebs which could be seen on photographs were counted. No blebs have been observed in the isolation solutions (phosphate-bicarbonate-sucrose) in any of the numerous preparations made. Blebs were observed to occur most frequently in 0.001 M KCl. Almost as many were seen in 0.001 M $MgCl_2$. In the latter instance the blebs were generally very large (Fig. 1) and in some instances

[1] The following solutions have been used for isolating nuclei in the present series of studies (22): *Solution I.* 0.0094 M KH_2PO_4, 0.0125 M K_2HPO_4, 0.0015 M $NaHCO_4$, 0.145 M sucrose. Solution II. Same salt concentration as Solution I, sucrose 0.218 M. *Solution III.* Same salt concentration as Solution I, sucrose 0.272 M.

TABLE I

Bleb Formation in Various Salt Solutions.
Results are expressed as number of blebs observed per 100 nuclei.

Solution	Molarity						
	1.0	0.5	0.25	0.15	0.05	0.01	0.001
NaCl	44	51	48	62	63	33	45
KCl	48	36	30	38	34	75	86
$CaCl_2$	0	0	13	7	46	69	73
$MgCl_2$	0	0	4	9	23	20	79
K-Phosphate (pH 7.02–7.37)	6	6	40	42	64	39	75

Double distilled water gave an average of 66 blebs per 100 nuclei, while none was observed in 3 times isotonic sucrose (0.88 M).

appeared to equal the nucleus in volume. A thicker component of the nuclear envelope appears to be invaginated beneath the blebs. When a preparation was observed for 20–30 minutes, many of the blebs could be seen to leave the nuclei as structureless spheres floating free in solution.

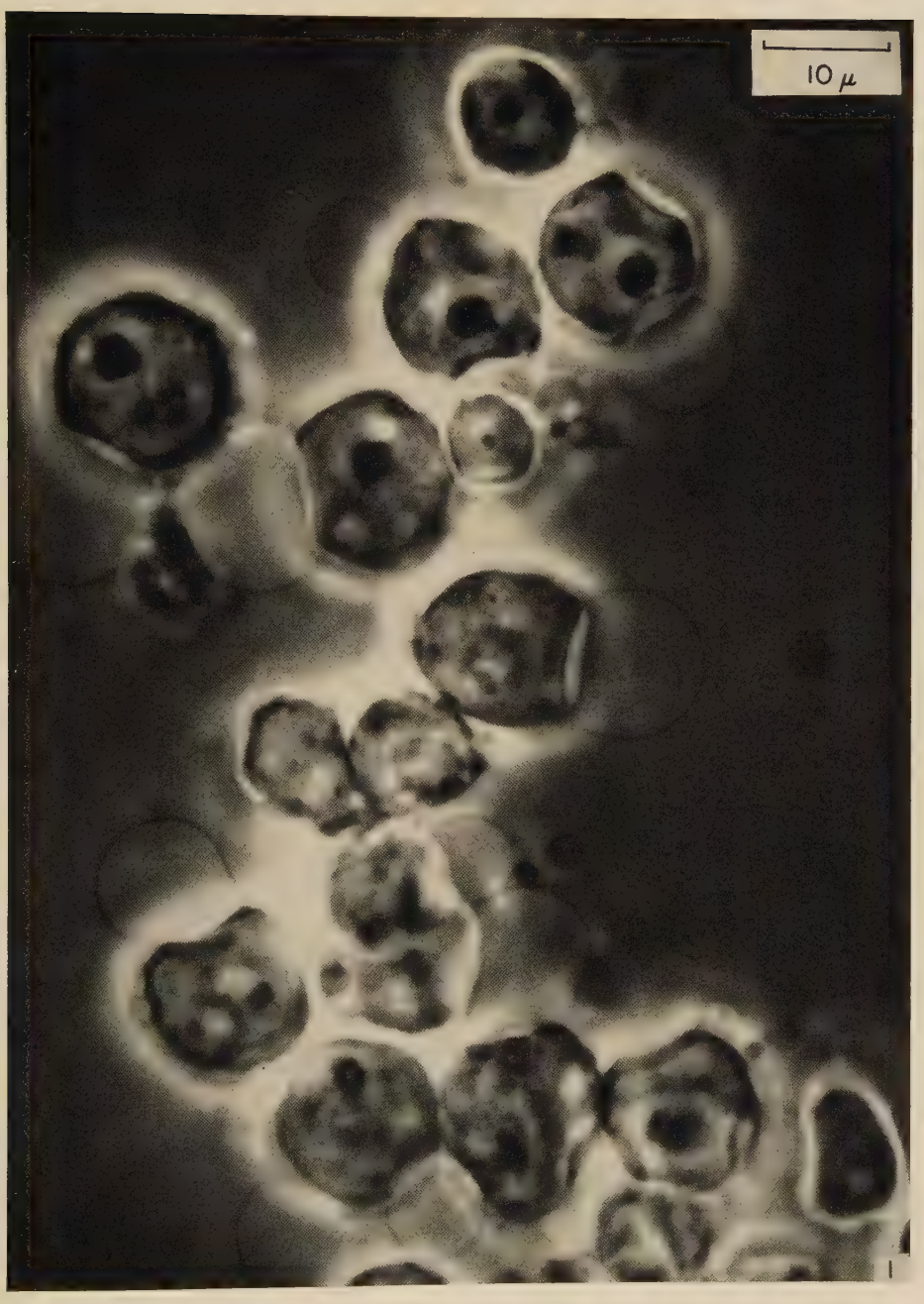
The formation of blebs was found to be a readily reversible phenomenon. Blebs formed by treating nuclei with 0.001 M $MgCl_2$ and which had not detached themselves from the nuclei, rapidly returned to the nuclear surface when the nuclei were replaced in Solution I (salt-sucrose isolation medium) (Figs. 2, 3). The reversibility of changes in nuclear volume and appearance in these solutions has been discussed in a previous publication (5).

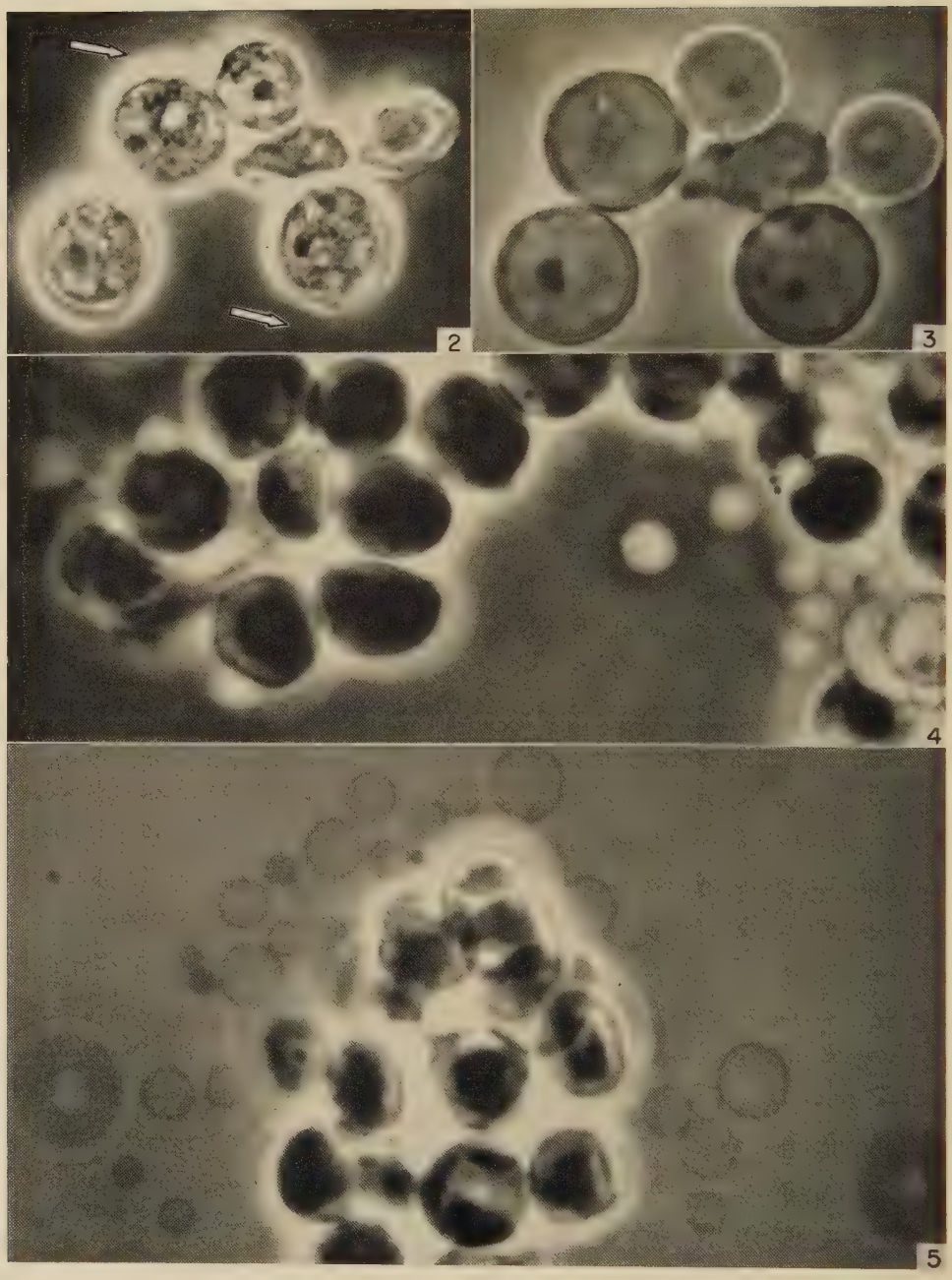
Observation has revealed blebs forming on nuclei inside the whole cells found in crude homogenates. However, it is not known whether these cells were uninjured.

The effect of sucrose on bleb formation. Bleb formation was observed when sucrose was omitted from the isolation medium (Solution I). No other change in either diameter, shape, or internal structure was seen. Reintroduction of the complete medium caused the blebs to disappear. The complete absence of bleb formation in the isolation medium is therefore due to the presence of sucrose.

The effect of sucrose in a number of concentrations (0.10, 0.05, 0.01,

Fig. 1. Isolated rat liver nuclei in 0.001 M $MgCl_2$. Note formation of numerous noncoalescing blebs and invagination of the thicker nuclear envelope component under the blebs. (American Optical Company dark medium phase contrast plates.)


10 μ


2
3
4
5

and 0.001 M) in 0.001 M $MgCl_2$ was studied to determine the range of concentrations over which sucrose prevented bleb formation, which was first evident in 0.01 M sucrose+0.001 M $MgCl_2$. Blebs in the latter solution were very small, however, and did not exceed 2 or 3 μ in diameter. In 0.001 M sucrose+0.001 M $MgCl_2$ large blebs were observed. It should be noted that sucrose appears to partially counteract the shrinking effect of very low concentrations of $MgCl_2$ on nuclei.

Observations on the permeability of nuclear blebs. The concentration of solutes inside the nuclear blebs appears to be very nearly the same as that in the various solutions used to produce bleb formation (distilled water, 0.001 M $MgCl_2$, 0.01 M KCl). This conclusion follows from the fact that the image produced in the phase contrast microscope is due to differences in index of refraction and, therefore, may be expected to reflect differences in physical density or solute concentration in the objects observed. If the nuclear blebs contained appreciable amounts of protein, for example, and were surrounded by a protein-free solution, they would appear dark against a gray background when viewed with dark-contrast phase objectives. Blebs impermeable to protein in a protein solution, on the other hand, should appear as bright structures against a gray background. The blebs described are difficult to observe directly, and when photographed with extremely high contrast photographic materials (Fig. 1) show neither appreciable density nor brilliance.

The addition of small amounts of substances which do not appear to penetrate the nuclear blebs results in a marked change in their appearance under the phase contrast microscope. As shown in Fig. 4, the blebs appear as light structures against a dark background when the nuclei are placed in a solution containing 5 per cent partially degraded gelatin[1] (molecular weight ca. 4 000) in 0.001 M $MgCl_2$. Similar results were obtained with bovine serum albumin. It appears from these observations

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Figs. 2 and 3. Same nuclei in 0.001 M $MgCl_2$ and in salt-sucrose medium (Solution I). Note complete reversibility of bleb formation and return to original homogeneous condition. Damaged nucleus in center shows changes similar to intact nuclei. Bleb-like series of faint concentric rings on edge of small nucleus to extreme right in Fig. 3 are due to small dense particle below the focal plane. (American Optical Company dark medium phase contrast plates.)

Fig. 4. Isolated rat liver nuclei 10 minutes after treatment with 5 per cent partially degraded gelatin in 0.001 M $MgCl_2$, showing formation of bright nuclear blebs. Note blebs which have come free of the nuclei. (Zeiss Winkle phase contrast plates, 1 470 ×.)

Fig. 5. Nuclei treated with 5 per cent partially degraded gelatin in Solution I, and then with 0.001 M $MgCl_2$. Note formation of numerous blebs which appear slightly darker than background. (Zeiss Winkle phase contrast plates, 1 470 ×.)

[1] A special preparation originally devised as a blood substitute.

that the blebs are not readily permeable to proteins. When fresh nuclei pretreated with Solution I plus 5 per cent partially degraded gelatin are placed in 0.001 M $MgCl_2$ plus 5 per cent partially degraded gelatin, blebs are again observed which appear as light objects against a gray background. However, when these blebbed nuclei are placed in 0.001 M $MgCl_2$, the blebs become first almost invisible, and then slightly darker than background as the gelatin in the solution around the nuclei is washed away (Fig. 5). This suggests that some gelatin entered the nuclei before the blebs were formed and later became incorporated in them, causing them to appear as slightly darker structures in a protein-free solution. Since this final density difference is not great, it is evident that only a small amount of protein entered the nuclei during the short period (6 minutes) before bleb formation was initiated.

As shown in Figs. 4 and 5 many blebs break away from the nuclei. Unfortunately the osmotic properties of these free blebs could not be studied, because introduction of different solutions under the cover slip washes them away.

All attempts to fix or preserve blebs for study with the electron microscope have so far been unsuccessful.[1] The introduction of fixatives or fat solvents causes the blebs to disappear. These observations, together with the evidence cited previously for a perinuclear lipid layer, suggest that the blebs may be composed chiefly of lipid material.

DISCUSSION

Blebs or blisters have been shown to occur on the surfaces of isolated rat liver nuclei in a variety of salt solutions and in distilled water. More were observed in dilute salt solutions, small ones being seen in very dilute solutions of salts of monovalent cations, while large ones were noted in very dilute solutions of salts of divalent cations. None have been observed in the phosphate-bicarbonate-sucrose solutions used for nuclear isolation in the present studies.

As has been previously noted, the envelope of the isolated rat liver nucleus appears to be permeable to a number of substances of relatively high molecular weight (heparin, highly polymerized DNA, and several proteins). It is unlikely, therefore, that the blebs are formed from a preexisting continuous surface sheet in response to changes in osmotic pres-

[1] Betty Martin, unpublished data — 1951.

sure. Rather, it is thought that the bleb-forming material (presumably phospholipid) is usually dispersed in the nuclear envelope. If the nuclear envelope is composed chiefly of a meshwork of protein fibrils or ribbons tangentially oriented for the most part, the bleb-forming material may be thought either to cover the fibrils or to be intimately associated with them. Such an envelope would allow free passage of high molecular weight substances. Under certain conditions (such as absence of sucrose and very low salt concentration), the bleb-forming material is thought to move to the surface, forming a continuous surface layer which may ultimately rise off to form blebs or blisters. As more of this material moves to the surface, the blebs are able to form complete spheres that break away.

The blebs do not appear to be readily permeable to protein, since blebs in 5 per cent solutions of bovine serum albumin or partially degraded gelatin show marked brilliance in the phase contrast microscope. Since the optical image under these circumstances reflects differences in refractive index (and therefore solute concentration), the fact that the blebs appear as luminous spheres against a dark background indicates that they have a lower refractive index than the surrounding medium, and therefore a lower concentration of protein. Blebs formed by nuclei previously treated with partially degraded gelatin appeared darker than the surrounding medium when placed in a protein-free solution, suggesting that in this instance some protein was trapped inside the blebs and could not readily escape.

It appears, therefore, that the blebs are less permeable than the envelope from which they are formed, and in contrast to the intact nuclear envelope, may behave osmotically. The mechanism of bleb formation, however, is not thought to be due to changes in osmotic pressure *per se*, since blebs have been observed to occur in a wide range of salt concentrations, in 5 per cent solutions of bovine serum albumin and partially degraded gelatin, and in distilled water. Sucrose (0.05 M) has been shown to prevent the appearance of blebs when added to a dilute $MgCl_2$ solution which otherwise caused extensive bleb formation. The inhibiting effect of sucrose does not appear to be due, necessarily, to its osmotic pressure. Rather, sucrose appears to have a rather specific effect in maintaining the dispersed state of the bleb-forming material. This conclusion is supported by 1) the observation that 0.145 M sucrose in 0.001 M $MgCl_2$ does not cause the blebs to shrink to a smaller size but rather causes a complete return to the nuclear surface, and 2) by the finding that bleb formation is prevented by a concentration of sucrose having approximately 1/5 the osmotic pressure of rat blood.

It is of interest to note that the mechanism proposed to explain bleb formation could also serve to produce cyclical changes in nuclear permeability.

Techniques for isolating rat liver nuclei in a condition morphologically similar to intracellular living nuclei still require the use of sucrose. It is not unlikely that nuclei isolated in salt solutions without sucrose have lost at least part of the bleb-forming constituents of the nuclear envelope.

SUMMARY

A number of salt solutions have been shown to cause tenuous blebs or blisters to rise from the surface of isolated rat liver nuclei. These have included solutions of NaCl, KCl, and K-phosphate (pH 7.0–7.4) in concentrations ranging from 0.001 M to 1.0 M, and $CaCl_2$ and $MgCl_2$ in concentrations ranging from 0.001 to 0.25 M. A large number of very small blebs were also observed in distilled water. The largest blebs were observed in dilute (0.001 to 0.01 M) solutions of $MgCl_2$ and $CaCl_2$.

The addition of sucrose in a concentration of 0.145 M was found to inhibit effectively bleb formation in a number of salt solutions, and to cause bleb retraction when added to bleb-forming solutions. Sucrose, in a concentration as low as 0.05 M, has been shown to prevent bleb formation by 0.001 M $MgCl_2$.

On the basis of index of refraction differences observed with the phase contrast microscope, it is concluded that the nuclear blebs are relatively impermeable to protein, but may contain protein which has previously penetrated the nuclear envelope.

A possible mechanism of bleb formation is presented.

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PHOTOMETRIC ESTIMATIONS OF DEOXYRIBONUCLEIC ACID IN INDIVIDUAL CELL NUCLEI

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The pioneer researches of Caspersson (4, 5, 6) on the application of quantitative ultraviolet microspectrophotometry to the study of cell structures, indicated the possibility of estimating the nucleic acid content of single nuclei with a reasonable degree of accuracy. The apparatus developed in his laboratory, however, is sufficiently complex and costly to place it out of the reach of most biologists. Considerably simplified photometric apparatus, as described by Pollister and Ris (51), Pollister and Moses (50) and by Lison (35), together with the use of the Feulgen and methyl green staining techniques, have greatly extended the use of the microscope as a quantitative tool in cytochemical studies on the nucleic acids.

The validity of ultraviolet microspectrophotometric procedures for the measurement of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) has been seriously challenged (9, 10), mainly on the grounds that molecular orientation of the nucleic acids may affect the measurements. These criticisms have been disputed by Pollister and Swift (52) and by Caspersson and Schultz (7).

The Feulgen reaction has long been used as a procedure for the detection and localisation of DNA within the cell. The basis of the reaction and its interpretation have been attacked by Stedman and Stedman (57, 58) whose criticisms have been countered by di Stefano (17), Li and Stacey (34), Ely and Ross (18), Overend (44) and Lessler (29). The reaction has been made the basis of quantitative measurements of DNA in single nuclei by several workers (1, 2, 3, 16, 20, 30, 32, 33, 36, 37, 38, 41, 42, 43, 45, 46, 47, 49, 53, 54, 55, 56, 60, 61).

The well-known affinity of nuclei for the dye-stuff methyl green has also been used for quantitative measurements of DNA, the specificity and stoichiometry of the reaction having been extensively investigated (15, 24, 25, 26, 27, 49, 62, 65). Kurnick (24) and Vercauteren (65) have emphasised that the ability of DNA to stain with methyl green is dependent on a high

degree of polymerisation, but evidence to the contrary has been presented by Taft (62).

A critical evaluation of possible errors in the application of microscopy to quantitative cytochemistry has recently been made by Glick, Engström and Malmström (19). It must be acknowledged that many of the criteria which they put forward as necessary conditions for valid microspectrophotometry have received but scanty attention in the past, and measurements on comparatively small numbers of nuclei have been credited with a higher degree of accuracy than the present development of the technique would appear to justify.

The present series of experiments was undertaken with a view to the comparison of the results obtained by the Feulgen, methyl green and ultraviolet techniques. Particular attention has been paid to the measurement of as many nuclei as practicable, from the same source of material in each case, and an attempt has been made to use one type of nucleus as a standard of comparison for both chemical and microscopic methods for the estimation of the mean DNA content per nucleus. In order to eliminate errors due to the measurement of cut nuclei in tissue sections, as well as those due to cytoplasmic absorption in the case of ultraviolet measurements, we have confined our observations to isolated nuclei from the adult rat and to nuclei in chick heart fibroblast cultures. We have also elected to use photographic, in preference to photoelectric, methods for the measurement of the extinctions of the nuclei, the possible lower accuracy obtainable thereby being fully offset, in our opinion, by the relative simplicity of the apparatus, the ease with which large numbers of nuclei can be recorded on film for measurement at leisure, and above all by the ease and speed with which data can be obtained in the ultraviolet region of the spectrum. Excessive irradiation of the specimen is also avoided.

METHODS

A) *Isolation of nuclei.*

Under ether anaesthesia, the liver and kidneys were rapidly removed from adult male albino rats. The organs were chilled in ice, and each was homogenised for 2 min. in M.S.E. Nelco blender, at top speed, in 4 or 8 vols. of an ice-cold solution of 0.25 M sucrose containing 0.008 Mcitric acid. The homogenates were strained twice through fine nylon gauze, and were then centrifuged at 0° for 10 min. at 880 g. The supernatant liquid was discarded, the precipitated nuclei and cell debris resuspended in 4

vols. of the citric acid-sucrose medium, and the mixture centrifuged for 6 min. at 500 g. The supernatant fluid was again discarded, and the precipitate resuspended and spun twice at 500 g. as before. Finally, the precipitate consisting almost entirely of cell nuclei, was resuspended and spun twice for 6 min. at 500 g in 0.01 M citric acid. Films of the resulting nuclei were made on glass and quartz slides, each slide having samples of both liver and kidney nuclei, and were allowed to dry in air.

The slides were fixed in acetic acid-ethanol (1 : 3 mixture) for 30 min. at room temperature and washed in running tap water for 2 hr. After drying at room temperature, they were stored in a desiccator till required, avoiding bacterial contamination.

B) *Tissue culture technique.*

Fibroblasts were obtained from embryonic chick heart implants by the roller-tube method (12), the cultures being grown on glass cover slips placed in contact with the walls of the roller tubes. After 96 hours, the dense central portion of each implant was removed under aseptic conditions, and growth was allowed to continue up to 120 hr. The resulting culture was in most cases of one-cell thickness and suitable for microscopy. The cover slips were removed, drained, immersed for 30 min. in the citric acid–sucrose solution described above, drained, fixed in acetic acid–ethanol for 30 min. and washed.

1 mm portions of 13-day chick embryo liver were placed in the citric acid-sucrose solution for 30 min., then smeared on one end only of glass slides, fixed as above and washed.

C) *Preparation of slides for microscopy.*

i) *Ultraviolet technique.* Since the cell nucleus contains, in addition to DNA, significant amounts of RNA, which will contribute appreciably to the ultraviolet extinction of the intact nucleus, it was decided to submit certain of the preparations to digestion with ribonuclease before ultraviolet photography. The ribonuclease employed was a crystalline preparation made by McDonald's (39) modification of Kunitz' (23) procedure. It had been shown by test to be free from proteolytic activity. The quartz slides were placed for 1 hr. in a solution containing 0.1 mg ribonuclease per ml of 0.02 M citrate buffer, pH 6.75, at 37°. They were then rinsed in buffer, dried in air, and mounted in 85 per cent glycerol-water mixture. The quartz cover slips were sealed with paraffin-wax, and slides were used within 3

hrs. of mounting. Control preparations were similarly mounted, without ribonuclease treatment.

ii) *Feulgen technique.* The leucofuchsin stain was prepared according to Coleman's (8) modification of de Tomasi's (14) method.

The slides were rinsed in cold NHCl, placed in NHCl at 60° for 6 min., again rinsed in cold NHCl, and transferred to water for a few seconds. They were then placed in the leucofuchsin solution for 2 hrs. at room temperature, drained, and transferred to the first of two jars containing the acid metabisulphite solution described by de Tomasi (14). After 30 min., the slides were transferred to the second metabisulphite jar for 30 min., thereafter being rinsed in running water, dried in air, and mounted in DPX neutral medium.

The cover slips carrying fixed tissue culture fibroblasts, and the slides carrying chick embryo liver smears, were immersed and stained simultaneously in the same jars, the cover slips being subsequently mounted on the unused ends of the slides.

iii) *Methyl green technique.* Attempts to stain smears of isolated nuclei by the method described by Kurnick (24), using a sample of methyl green kindly supplied by him (National Aniline Corpn., Cert. No. 26, C.I. No. 685), were disappointing in our hands, since the staining was so faint as to be barely detectable. The use of neutral formalin for fixation did not affect the staining, and attempts to stain deparaffinised or frozen sections of whole liver by this method were equally unsuccessful, irrespective of the fixative employed.

Satisfactory staining was obtained by the method described by Pollister (48), the dry stain or its aqueous solution having been exhaustively extracted with chloroform to remove contaminating methyl violet before being diluted with the phenol-glycerol-ethanol-water mixture. Similar results were obtained with either methyl green marketed by the National Aniline Corpn., or methyl green marketed by Gurr, batch no. 3524, which are understood to be ethylated hexamethylpararosaniline and heptamethylpararosaniline respectively. (It is worthy of note that many commercial samples labelled "methyl green" consist of a quite different dyestuff, with an absorption spectrum similar to that of true methyl green, but differing from it in being freely soluble in chloroform, as well as differing in staining properties and specificity.)

The stained slides were dried in air and mounted in DPX medium. Photography was done immediately, although we have not experienced difficulty due to fading of the stained preparations.

D) *Apparatus for microscopy.*

Photomicrography was carried out with a type M 8001 ultraviolet microscope manufactured by Cooke, Troughton and Simms, Ltd. For ultraviolet photography of unstained preparations, the light source was a quartz low-pressure mercury resonance lamp, the 254 mμ Hg line being isolated by a single quartz prism. The Feulgen-stained preparations were photographed at 546 mμ, a high-pressure mercury arc being used in conjunction with a colour filter (Hilger type "mercury green") and a single prism. The methyl green preparations were photographed using a "Pointolite" lamp together with Ilford filter type 607 plus Chance filter OR2, giving peak transmission at 630 mμ. In all cases, the Kohler principle of illumination was used.

The optical systems were:—

for ultraviolet: 2 mm fused quartz objective, corrected for 254 mμ, N.A. 1.25, immersion liquid 85 per cent glycerol; 10× quartz ocular, ocular-to-film distance 22 cm;

for Feulgen and methyl green: 3.75 mm fluorite objective, N.A. 0.95, immersion liquid 85 per cent glycerol; 10× glass ocular, ocular-to-film distance 22 cm.

The substage condenser was quartz, glycerol immersion, N.A. 1.25, iris aperture 6 mm.

Camera: 35 mm Leica without lens, and interchangeable with either ground glass or fluorescent focussing screens, or with a fluorescent eyepiece.

On each strip of film there were photographed from 10 to 20 fields of liver nuclei, and 10 to 20 fields of kidney nuclei from the same slide, or equivalent numbers of fibroblast and chick embryo liver nuclei when appropriate. A logarithmic step-wedge was then photographed on to each strip of film, by placing the camera immediately behind a rotating step-sector in the same light path as that used for the microscope. The exposure for the wedge was chosen by trial and error, to give a suitable range of negative densities.

Film: Ilford type FP3, development 10 min. in Kodak D76 developer at 18°.

E) *Measurement.*

The nuclei were individually numbered on the negatives, those which overlapped or were obviously damaged being rejected. The negatives were

projected to 20 diameters enlargement, and their outlines drawn, the area of each nucleus being measured, in arbitrary units, by a planimeter. The negatives were measured by a Hilger spectrograph plate densitometer, galvanometer deflections being recorded for each step of the step-wedge, for three small areas in the central part of each nucleus, and for two equal areas of "empty" background adjacent to each nucleus.

F) *Calculation of amounts of absorbing material in each nucleus.*

From the step-wedge readings, a graph was drawn of galvanometer deflections against film exposure. From this graph, readings were taken for the intensity (T) of light transmitted through the centre of each nucleus and the intensity (I) of light reaching the film adjacent to each nuclear image, these values being given by the corresponding galvanometer deflections for the portions of the negative representing the central core of the nucleus, and the area adjacent to the nucleus, respectively.

The Extinction (E) of the central core of the nucleus is given by

$$E = \log_{10} \frac{I}{T}$$

and in spherical or ellipsoidal nuclei the amount of absorbing material present is taken as being proportional to the product of Extinction × Area.

This calculation is applicable equally to the estimation of ultraviolet absorbing material, and of the amounts of Feulgen or methyl green dye fixed in each nucleus. The units, however, are purely arbitrary, and are not directly comparable as between the different techniques.

RESULTS

Figs. 1 to 3 present, in the form of frequency histograms, the data which we have obtained by the photometric analysis of batches of liver and kidney nuclei.

Fig. 1 shows the distribution of the DNA content of nuclei in rat kidney and liver, measured in terms of the amount of Feulgen dye fixed in each nucleus. The values for kidney nuclei form a reasonably symmetrical peak about a mean of 443 units, and while there is a considerable scatter of values, extending from about 300 to 600 units, only a very few measurements fall outside these limits (less than 5 per cent of the total). In the liver nuclei from the same animal, however, the range of values is very much wider. Liver nuclei clearly fall into two main groups; a smaller peak with

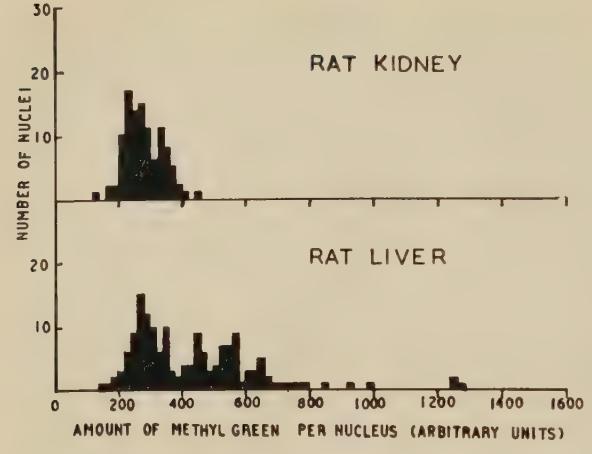

Fig. 1. DNA content of kidney and liver nuclei, in arbitrary units, measured by the Feulgen reaction. Male albino rat, wt. 260 g. *Upper chart:* 224 kidney nuclei, mean DNA content 443 units. *Lower chart:* 234 liver nuclei, mean DNA content 830 units, *comprising* 61 nuclei, containing 0–650 units, mean 443, 165 nuclei, containing 650–1300 units, mean 941, 8 nuclei, containing 1300 or more units, mean 1597.

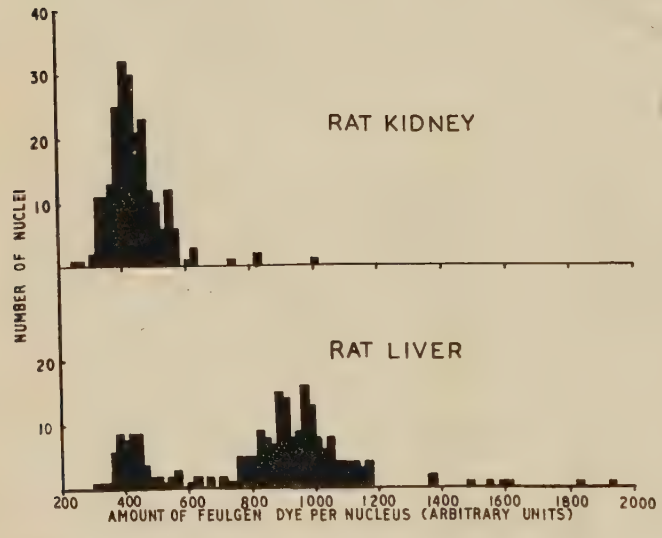

Fig. 2. DNA content of kidney and liver nuclei [Methyl green staining]. Male albino rat wt. 370 g. *Upper chart:* 106 kidney nuclei, mean DNA content 278 methyl green units. *Lower chart:* 159 liver nuclei, mean DNA content 436 methyl green units, *comprising* 80 nuclei, containing 0–400 units, mean 288, 73 nuclei, containing 400–800 units, mean 545, 6 nuclei, containing 800 or more units, mean 1091.

a mean value, again 443 units, apparently corresponding to that of the kidney nuclei, and a larger and more scattered group with a mean value of 941 suggesting a DNA content slightly more than double that of the nuclei in the lower peak and in the kidney. A few liver nuclei present much higher values.

Methyl green staining of nuclei prepared under the same conditions as those shown in Fig. 1 but from a different animal gave the results shown in Fig. 2. Once again a similar picture emerges, with the kidney nuclei

CORRECTION

Frazer, S. C., and Davidson, J. N. Photometric estimations of deoxyribonucleic acid in individual cell nuclei. *Exptl. Cell Research*, **4**, 316 (1953).

On p. 322 the figures have been interchanged. Figure 1 and legend refer to the lower diagram; Figure 2 and legend refer to the upper diagram.

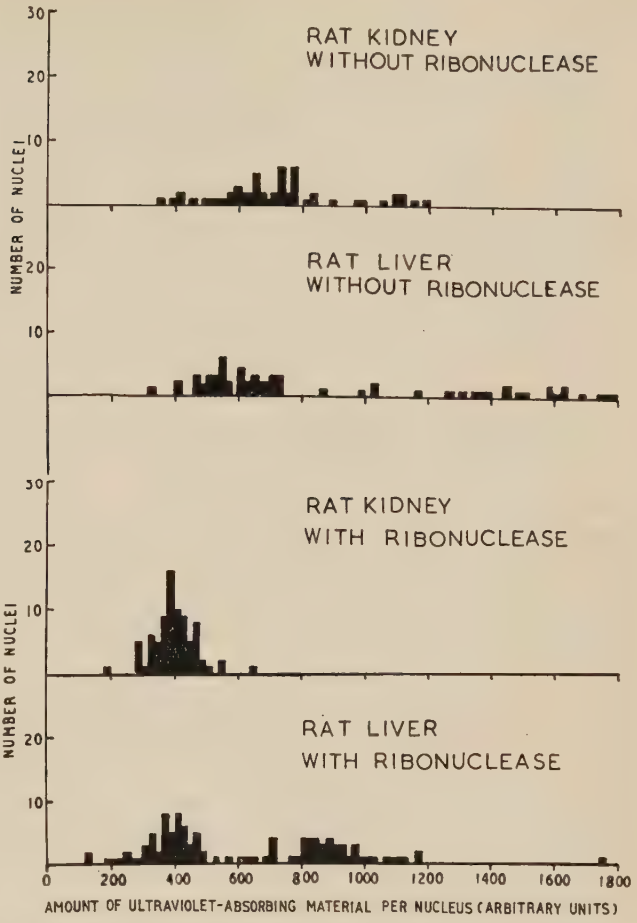

Fig. 3. Ultraviolet absorbing material in kidney and liver nuclei [$\lambda = 254$ mμ]. Same batches of nuclei as Fig. 2. A. Without ribonuclease. *Upper chart:* 55 kidney nuclei, mean 725 units. *Lower chart:* 63 liver nuclei, mean 889 units. B. With ribonuclease treatment. *Upper chart:* 81 kidney nuclei, mean 382 units. *Lower chart:* 105 liver nuclei, mean 611 units, *comprising* 58 nuclei, containing 0–600 units, mean 376, 46 nuclei, containing 600–1200 units, mean 881, 1 nucleus, containing 1760 units.

all falling into one category. The distribution curve of the values for liver nuclei again shows a definite peak with a mean value the same as that of the kidney nuclei, while there is a second peak with a mean approximately double that of the lower peak. A few nuclei again have much higher values. The over-all picture presented by the methyl green stained nuclei, however, is less well-defined than with the Feulgen preparations.

The measurements of ultraviolet absorbing material at 254 mμ are given in Fig. 3, the upper section representing kidney and liver nuclei not treated with ribonuclease, while the lower section gives data from nuclei of the same batches after incubation in a solution of crystalline ribonuclease.

In the former case, the measurements represent all substances absorbing ultraviolet light at 254 mμ, including DNA and RNA. After ribonuclease treatment, the only absorbing substance remaining in significant amounts should be DNA, and the data in the lower half of Fig. 3 should give the relative amounts of DNA present in the different types of nuclei, subject, of course, to the errors of measurement. Where the ultraviolet absorbing material is measured without ribonuclease treatment, the values for both kidney and liver show a very wide scatter, and while the mean value for all the liver nuclei in this specimen is higher than that for kidney nuclei, there is no definite tendency for the liver nuclei to fall into two distinct groups. The scatter of the kidney values is very much greater than in any of the other series of measurements. Fig. 3 b, however, presents a different aspect; following ribonuclease treatment, the mean value for the kidney nuclei has fallen from 725 units to 382 units, and the distribution has become similar to that found by Feulgen or methyl green staining. In liver too, the ribonuclease treatment reduces the mean value per nucleus from 889 to 611, and the two main groups of nuclei again become apparent. One peak, having a mean value of 376, again corresponds to the mean value found for kidney nuclei while the higher peak, though somewhat flattened, gives a mean of 881 units.

It is evident that ribonuclease treatment removes a considerable amount of ultraviolet absorbing material from kidney and liver nuclei, and that the amount of absorbing material per nucleus which remains appears to correspond, in a population of nuclei, with the ability of similar nuclei to stain with either Feulgen or methyl green dye. Also, the results imply that the material removed is of much more variable distribution amongst the nuclei than is DNA itself.

We have also considered briefly the classification of nuclei in terms of their size, and attempted to correlate the size of each nucleus with its content of DNA as measured by photometric methods. Fig. 4 indicates the relationship between nuclear area and DNA content, for the liver nuclei recorded in Fig. 1, and the results for the methyl green and ultraviolet techniques are similar. It is clear that while the small liver nuclei (derived from bile ducts, Kupffer cells, blood vessels and the like) all correspond to the kidney nuclei in having a DNA content of about 400 units, the larger nuclei from the liver parenchyma cells fall almost entirely into the higher group of values. Only a few large liver nuclei have a low DNA content. The very large nuclei sometimes encountered all have high DNA values.

In any attempt to correlate the DNA content of liver and kidney nuclei,

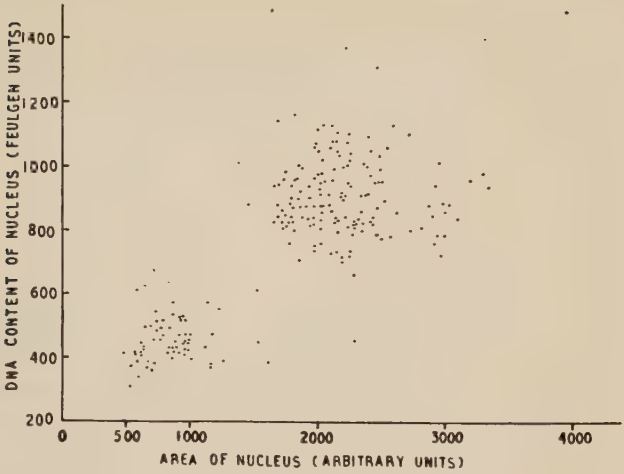

Fig. 4. Relationship between nuclear size and DNA content, in rat liver. DNA content in arbitrary Feulgen units. Size expressed as arbitrary units of area. [Measurements on the same nuclei as those of Fig. 1.]

as found by photometric methods, with the mean values obtained from nuclei in bulk, by ordinary chemical procedures, we are faced with the probability that the batches of nuclei used in each case are not valid samples as regards the relative frequency of occurrence of each type of liver nucleus. Such factors, for example, as the relative fragility of nuclei from different types of cell, might lead to selection of one type for measurement, and so to excessively high or low mean values for liver nuclei, when compared with the values for kidney. We have therefore carried out differential counts on sections of adult rat liver, and made tentative comparisons on the assumption that the lower liver peak represents small nuclei, while the higher peak represents parenchyma cell nuclei. Although these comparisons can be only partially valid, since some true parenchyma cells have a value lower than that of the higher peak, the fact that there are also parenchyma cells present whose nuclear DNA is higher than the mean of the higher peak will go some way towards correcting the error.

Our differential counts indicate that about 58 per cent of the cells present in sections of adult rat liver are parenchymal, the remainder being bile duct epithelium, Kupffer cells, blood vessel walls, and the like.

The figures quoted by other authors (40, 59) for the relative proportions of diploid and polyploid cells in liver refer to histological observations on hepatic cells only, and are not therefore of assistance in calculations based on estimations on isolated nuclei. Our samples contain nuclei of all types occurring in liver, and the frequency of occurrence of polyploidy in our mixed nuclei will not necessarily correspond to that in hepatic cells alone.

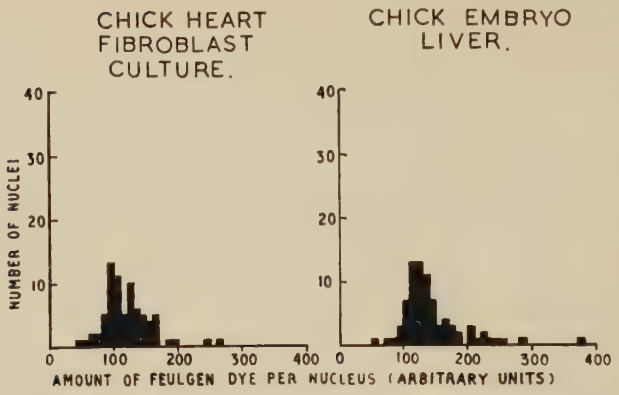

Fig. 5. DNA content of chick heart fibroblast nuclei and chick embryo liver nuclei (Feulgen units). *Upper chart:* 74 fibroblast nuclei, mean 120 units. *Lower chart:* 80 liver nuclei, mean 145 units, *comprising* 69 nuclei, containing 0–200 units, mean 129 units, 11 nuclei, containing 200–400 units, mean 244 units, (120 hour roller-tube fibroblast culture; 13 day chick embryo liver.)

The ratio of the mean amount of DNA per liver nucleus to that per kidney nucleus calculated from the data presented in Fig. 1, is 1.65 for photometric estimations on the assumption that the great majority of the liver parenchyma cell nuclei fall into the higher group of values, while the bile duct nuclei and those of the blood vessels and sinusoids fall into the lower group. The corresponding figure from direct chemical estimations on isolated nuclei in bulk is 1.40 (11). The discrepancy may be attributable not only to errors in both methods of estimation, but also to variations in the differential count in different animals as well as to possible selective isolation of nuclei from one or other cell type prior to chemical estimation.

Fig. 5 presents data obtained from tissue cultures of embryo chick heart fibroblasts and smears of chick embryo liver, by photometric estimations following Feulgen staining of the cell nuclei in these preparations. The DNA content of fowl nuclei is considerably lower than that of rat nuclei (42, 13).

In the fibroblast culture, the mean value of 120 units is slightly lower than the over-all mean value for chick embryo liver nuclei (145 units). In each case, the distribution curve is asymmetrical, there being few values below the peak, and many of the nuclei give values approaching twice that of the peak. If the peak frequency value is regarded as corresponding to a "diploid" chromosome set, the data are consistent with the presence, in these rapidly growing cell populations, of many nuclei with DNA values

intermediate between "diploid" and "tetraploid." In the liver smear there is an appreciable number of nuclei with DNA values of approximately twice that of the mean of the main group, although the proportion of such nuclei is very much smaller than is found in adult rat liver.

DISCUSSION

In the application of microspectrophotometry to chemical analysis of biological specimens where information is being sought which is quite unobtainable by normal chemical methods, it is probably justifiable to accept a much greater error than is normally permissible. It is however essential to minimise experimental error by surveying the results as a whole. We have accordingly been careful to make as many observations as possible on each batch of material, and to avoid drawing any conclusions from the measurement made on any one nucleus.

The question of the specificity of the Feulgen and methyl green staining techniques, and of the stoichiometry of the reactions involved has been extensively discussed by other authors (26, 27, 48) and we do not propose to add any fresh evidence from the chemical aspect. The theory of ultraviolet absorption measurements is well known also (6, 7) and need not be further discussed at this point.

The influence of the ultraviolet absorption due to proteins in isolated nuclei is so small as to be negligible at the wavelengths used for measurements of nucleic acids (31) but the view that the influence of nuclear RNA is altogether negligible (31) is not in agreement with our data in Fig. 3, which indicates that ribonuclease removes a considerable proportion of the total material absorbing in the region 250–260 mμ. Although part of this RNA may represent cytoplasmic contamination, our preparations show little microscopic evidence of cytoplasmic debris adhering to nuclei.

In any case, the influence of both nuclear RNA and cytoplasmic contamination can successfully be eliminated by treatment with crystalline ribonuclease.

The side-by-side comparison of the results obtained by Feulgen, methyl green, and ultraviolet techniques reveals a sufficiently close agreement in the pattern of distribution of the material measured in rat liver and kidney nuclei to support the view that the three methods are measuring substantially the same substance. If the arbitrary units given by the photometric measurements are to be converted into absolute units of DNA, however, considerable caution must be used since there is evidence (28) that the

Beer-Lambert laws are not fully valid when applied under the conditions inherent in these photometric procedures. The accuracy of photometric observations falls off considerably, also, when very low or very high extinctions are being measured. The values expressed in terms of our three types of arbitrary unit may not be strictly proportional to the absolute amounts of DNA present but the deviation from proportionality is not likely to be excessive except where the nuclei are exceedingly small and dense, or exceedingly large and diffuse, and these represent only about 5 per cent of the total measured. The pattern of different nuclear types found in rat liver cannot be explained by divergence from the Beer-Lambert laws.

The method we have employed in calculating the amount of absorbing material in a nucleus requires in theory that the nucleus shall be spherical or at least regularly ovoid. While many workers have on this account confined their measurements to nuclei which they judged to be spherical, we have in the first instance measured all intact nuclei without regard to shape. If we reject from our results all measurements made on ovoid or irregular nuclei, the amount of numerical data available for study is greatly reduced but the general pattern of results is unaltered and the scatter of the peaks is not reduced. We have accordingly presented the data on all the nuclei measured. (Difficulties in measurement of both extinction and area of chromosomes render untrustworthy the application of the method to nuclei in the course of mitosis, although results obtained in this way have been used to support a hypothesis of the relationship between mitosis and changes in DNA (36, 37)).

For accurate photometric measurements the distribution of absorbing substances in the nucleus should be as uniform as possible. The reticular structure of "chromatin" given by most fixatives can be converted to a satisfactory homogeneous dispersion by the use of solutions of sucrose prior to fixation (55). We have also taken the precaution of making three measurements near the centre of each nucleus, and calculating the extinction from the mean of the three.

Measurements on isolated nuclei, rather than on tissue sections, eliminate the possibility of erroneous measurements on sliced nuclei, and adjacent clear areas on the slide are readily available for incident light measurements. The possibility that DNA may be lost during the course of isolation is minimised by the inclusion of citric acid in the isolation medium. It is also probable that a batch of nuclei isolated from, say liver, may not represent a true sample of the relative proportions of the different types

of cell present. The effect of selection is less important when photometry is employed but will have an important effect on the mean DNA value per nucleus found by chemical methods, particularly in polyploid tissues such as liver.

Although orientation of the DNA molecules within the nucleus may in theory introduce possible errors into ultraviolet absorption measurements (9, 10), a comparison of our results by the ultraviolet method, with those by either methyl green or Feulgen staining, which are presumably immune from molecular orientation, does not suggest that the orientation is producing any significant error.

Chemical methods show that in adult, non-dividing tissues from the same species the mean amount of DNA per nucleus falls within comparatively narrow limits, the somatic nuclei having approximately double the DNA content of sperm heads (42, 63, 64). This finding has to some extent been misinterpreted as implying a physical constancy of the amount of DNA in every non-dividing nucleus, although the chemical methods employed can only provide a mean value for the nuclei isolated from a particular tissue. While photometric methods do not yet allow of the accurate comparison of individual nuclei sufficient evidence has accumulated to show that nuclei from homogeneous cell populations such as sperm or erythrocytes do in fact show a very similar DNA content. Our findings in rat kidney nuclei are consistent with the general hypothesis that such nuclei, although coming from a range of cells with widely varying functions, all contain substantially the same amount of DNA.

An exception to the general constancy of the nuclear DNA in different tissues is the observation that rodent liver regularly gives mean DNA values considerably higher than those of other tissues in the same species. Swift (60), using the Feulgen reaction on mouse liver nuclei, showed that the high mean value represented the effect of admixture of two distinct types of nuclei with about twice and four times the sperm DNA content respectively. Some nuclei showed higher values still. Our findings in rat liver by all three methods agree substantially with those of Swift, and confirm that the high mean value does not imply any departure from the general hypothesis outlined above. That rodent liver contains a considerable proportion of polyploid cells is known from other evidence (22, 67), and our evidence, like that of Swift (60, 61) supports the concept of diploid somatic cells in most tissues, with a considerable proportion of tetraploid cells in adult liver. Our information further suggests that in the adult albino rat, the majority of the true hepatic cells are in the presumed tetraploid category,

while the concomitant structural and vascular tissue cells in liver are almost exclusively "diploid" as regards DNA content.

The measurements reported on chick embryo heart fibroblasts, and on chick embryo liver, present a somewhat different picture from that found in adult tissues. The cells in each case form a group, in which most of the nuclei have the same DNA value, but in each case there are cells present apparently showing values higher than the presumed diploid peak but lower than the "tetraploid" value. (No measurements were attempted on cells undergoing visible mitosis.) The findings are at least not inconsistent with the widely held view (1, 21, 53, 66) that synthesis of DNA occurs during interphase, building up to more than the resting value prior to cell division.

SUMMARY

1. The deoxyribonucleic acid content of individual cell nuclei has been measured by microspectrophotometry of nuclei isolated from rat liver and kidney, and of nuclei in chick heart fibroblast cultures and chick embryo liver smears. Measurements have been made on nuclei stained by the Feulgen and methyl green methods, and on unstained nuclei by ultraviolet absorption methods, with and without ribonuclease pretreatment.

2. The method does not permit of absolute measurements of the DNA content of single nuclei, but side-by-side measurements on large numbers of nuclei from two tissues allow their nuclear populations to be compared.

3. Ribonuclease digestion removes substantial amounts of ultraviolet-absorbing material from rat liver and kidney nuclei. Following ribonuclease treatment, ultraviolet measurements present a general picture corresponding to that found by measurement of Feulgen- and methyl green-stained material.

4. Adult rat kidney nuclei form an approximately homogeneous population as regards DNA content, while liver nuclei from the same animal form two main classes, one with the same DNA content as kidney nuclei, and one with approximately twice that amount of DNA. A few nuclei have still higher values.

5. The majority of the nuclei present in chick heart fibroblast cultures have DNA values of the same order as those found in chick embryo liver. In each case, nuclei are found whose DNA content approaches twice that of the majority of the nuclei present.[1]

[1] Dr. I. Leslie of this Department has recently shown by chemical analysis that the mean DNA content of nuclei isolated in bulk from chick heart fibroblast cultures is of the same order as that of similarly isolated nuclei from embryonic chick heart and liver.

We wish to thank Dr. I. Leslie for preparing the tissue cultures used in these experiments, Dr. N. B. Kurnick for supplying us with a specimen of methyl green, and Dr. S. Mitchell of the Chemistry Department of this University for allowing us the use of a densitometer.

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ON THE IDENTITY OF "ISOLATED CHROMOSOMES"[1]

II. DISTORTED NUCLEI IN THE PRODUCTION OF CHROMATIN THREADS

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Conflicting views exist on the identity of "isolated chromosomes" (*cf.* 3, 5, and 6). This question is fundamentally important in cancer research. In a previous paper (1), the forms and sizes of chromatin threads isolated from chicken erythrocytes by two radically different means were found strikingly similar to those of mitotic chicken chromosomes. Their fine-structure also recalled the structure of chromosomes. This initial evidence thus indicated that these isolated threads indeed represented the chromosomes.

We consider here the role of distorted nuclei in the production of chromatin threads. Lamb (2, 3) concluded that various cells (chiefly calf thymus) were commonly affected by several mechanical means of disruption (including the Waring Blendor) by "drawing" of the nuclei into threads of fragmentary character that bore "little resemblance" to chromosomes. Ris and Mirsky (5) indicated that distorted nuclei were seen only in small numbers in their preparations. This disparity on a point crucial to the conclusions seemed to demand further examination. Serial results of such preparations with the Waring Blendor (*WB*) and the Logeman Mill (*LM*) are reported here. Correlating evidence is also given from studies of whole cells not subjected to any such mechanical treatment.

SERIAL PREPARATIONS WITH THE WARING BLENDOR AND THE LOGEMAN MILL

Working generally as before (1) with erythrocytes of domestic chickens (Plymouth Rock), specimens of serial mixes were mounted for microscopic examinations after treatments of 1/2, 1, 1 1/2, 2, 4, 8, and 16 minutes in the Waring Blendor and after each of 10 extrusions in the Logeman Mill. The former series fortuitously but happily coincides with that employed by Lamb (3). The final resedimented products were

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TABLE I

Disruption of chicken erythrocytes by Waring Blendor and by Logeman Mill.[1]

Waring Blendor			Logeman Mill		
Time of Treatment, minutes	N/C[2]	T/N[2]	Number of Extrusions	N/C[2]	T/N[2]
1/2	.8	.5	1	5	0
1	4.4	1.0	2	6	.7
1 1/2	6.0	1.2	3	55/0	1.8
2	19	1.4	4	37/0	3.3[3]
4	21/0	4.8[3]	5	24/0	4.5[3]
8	17/0	8.4[3]	6	39/0	3.3[3]
16	28/0	11[3]	7	35/0	5.0[3]
			8	30/0	5.3[3]
			10	28/0	8.0[3]
Final product:	32/0	11[3]	Final product:	10/0	8.4[3]

also mounted. Gey's solution (4) less the dextrose, hereafter referred to as "Gey's salt solution", was used as medium during these preparations; the pH was 7.8. Wetmounts of the fresh material were made for phase microscopic examination. Permanent slides for light microscopy were made with Wright's, acetocarmine, or Feulgen staining. Mounts of the same materials for electron microscopy were made for all specimens either unfixed or bulk-fixed with equal volumes of acetocarmine or osmium tetroxide for at least 10 minutes; final products were also prepared similarly with fixations by acetic alcohol or chromic acid.

Phase microscopy permitted detailed qualitative descriptions, systematic counts, and micrographs that indicated the progressive and successive disruption of the cells and nuclei to produce characteristic threads (Table I). The parameter N/C (number of free nuclei per intact cell) increased rapidly and regularly in both instances. Cells were absent after 2 minutes of treatment with the *WB* and after 2 extrusions in the *LM*. The ratio T/N (number of free threads per free nucleus) likewise increased rather regularly in both instances, but more slowly. This trend is believed real despite the presence of tangles in the later specimens that precluded the adequate enumeration of threads. Threads of characteristic appearance were seen after the shortest treatment with the *WB* (1/2 minute) and after but 2 extrusions in the *LM*.

[1] From systematic counts of fresh wetmounts by phase microscope. Details of treatment and scoring given in (1).

[2] C denotes number of intact cells, N the number of free intact nuclei, T the number of discrete free threads.

[3] Tangles of threads were present; these ratios are thus very conservative.

TABLE II

Phase-microscopic counts of distorted nuclei in serial preparations by the Waring Blendor.

Time of Treatment, minutes	Number of Nuclei		Proportion of "Drawn" Nuclei[1]
	"Drawn"[1]	Total	
1/2	2	80	.025
1	0	33	.00
1 1/2	2	50	.04
2	2	80	.025
4	1	31	.03
8	0	27	.00
16	3	83	.036
Totals	10	in 384	mean .026

Standard deviation from overall mean, .017. Standard error of overall mean, .006. Maximum uncorrected $t=1.5$; no significant differences of individual proportions from the overall mean.

The nuclei seen in these fresh wetmounts were characteristically round. The proportion of distorted or "drawn" nuclei throughout the *WB* treatment was statistically constant at 2.6 per cent with a standard error of 0.6 per cent (Table II). Only 2 distorted nuclei were seen in 534 counted through the ten extrusions by *LM*; a much greater total number of nuclei was seen in scanning without further observation of distortion.

The fixed slides examined in the light microscope showed Feulgen-positive meshes of threads and persisting intact nuclei.

In the electron microscopy, which included about 1 200 individual micrographs, cells were absent after 3 minutes of treatment in the *WB* and after 2 extrusions in the *LM*. Threads of characteristic forms, sizes and electron-opacity appeared after 1/2 minute of treatment in the *WB* and after but 1 extrusion in the *LM*. The many threads in both types of material included a variety of forms. Many resembled mitotic macrochromosomes. Doubleness and axial variations in width and in electron-opacity were seen. Some atypically long or heavy threadlike objects were occasionally observed in both types of preparations. Fig. 1 is an example of the material, representing the mix after 7 extrusions in the *LM*. This includes a round nucleus, a typical thread measuring about $1/2 \times 5$ micra that displays in original prints a clear axial variation in electron-opacity, and a thread measuring about $1/3 \times 10$ micra.

[1] Reference is made here to all nuclei that were importantly distorted; those scored all possessed tapering bodily protrusions, in contrast to the round appearance of most of the nuclei.

TABLE III

Distributions of nuclei by type as seen in the electron microscope: preparations with the Waring Blendor.

Types of nuclei distinguished: *O*=essentially *rounded*, *D*="drawn", with typically tapering, massive protrusions, *T*=nuclei with *threadlike*, nontapering protrusions.

Time of treatment, minutes	Numbers of Nuclei of Type				Proportions in tenths		
	O	*D*	*T*	Total	*O*	*D*	*T*
1/2	9	2	5	16	.6	.1	.3
1	12	5	18	35	.3	.1	.5
1 1/2	5	3	7	15	.3	.2	.5
2	1	1	5	7	.2	.2	.7
4	6	5	11	22	.3	.2	.5
8	2	5	17	24	.1	.2	.7
16	9	6	20	35	.3	.2	.6
Totals	44	27	83	154			
Average Ratios[1]	.29	.18	.54				
Final Products:							
Acetocarmine ...	2	4	11	17	.1	.2	.6
Osmium tetroxide	6	2	10	18	.3	.1	.6
Chromic acid	2	1	10	13	.2	.1	.8
Acetic alcohol ...	2	0	0	2			
Totals	12	7	31	50			
Ratios	.24	.14	.62				

Nuclei seen in the electron microsope were characteristically distorted. Two types were apparent, as mentioned by Lamb (3), that may be illustrated by a few selected micrographs: Some nuclei, possibly "drawn", appeared with massive and usually tapering bodily protrusions (Figs. 2, 3, and 4). Others occurred with nontapering threadlike extensions characteristically less than 1/2 micron in diameter (Figs. 5, 6, and 7).[2] Some nuclei showed both types of protrusion (Figs. 8 and 9). Double or filamentous structure was sometimes apparent in these protrusions (e.g., Figs. 3 and 8), even in

[1] Standard deviations are .15, .06 and .12 for the individual serial proportions of types *O*, *D* and *T*; maximum uncorrected *t* is 2, showing no significant differences of the serial values from the overall means. Standard errors of overall means are .057, .023 and .045 for types *O*, *D*, and *T* respectively.

[2] Lamb (3) examined such nuclei with apparently emerging threads and reported that all appeared to represent threads attached to the nucleus rather than emerging threads. However, such objects found here will be referred to as "protrusions" because of (a) their appearance in the electron microscope and in the micrographs, (b) their observed movement along with the

TABLE IV

Distributions of nuclei by type as seen in the electron microscope: preparations with the Logeman Mill.

Types distinguished: round (0), "drawn" (D), and with threadlike protrusions (T), as in Table III.

Number of Extrusions	Numbers of Nuclei by Type				Proportions in tenths		
	O	*D*	*T*	Total	*O*	*D*	*T*
1	29	2	8	39	.7	.05	.3
2	10	2	19	31	.3	.06	.6
3	8	5	13	26	.3	.2	.5
4	16	4	18	38	.4	.1	.5
5	9	5	14	28	.3	.2	.5
6	14	3	14	31	.5	.1	.5
7	5	6	19	30	.2	.2	.6
8	2	4	12	18	.1	.2	.7
9	7	6	18	31	.2	.2	.6
10	7	3	14	24	.3	.1	.6
Totals	107	40	149	296			
Average Ratios[1]	.36	.14	.50				
Final Products:							
Acetocarmine ...	6	6	8	20			
Osmium tetroxide	13	5	18	36			
Chromic acid	1	1	9	11			
Acetic alcohol ...	3	6	17	26			
Totals	23	18	52	93			
Ratios	.25	.19	.56				

the heavier masses (Fig. 10). Evidence of axial variations in their electron-opacity was also seen (Figs. 7 and 8).

Counts made from the many representative electron micrographs provided interesting data. Thus, 71 per cent and 64 per cent of nuclei were found with apparently associated protrusions for materials prepared by the *WB* and the *LM*, respectively (Tables III and IV). These figures are to be contrasted with

nuclei in several instances of rupture of the supporting film in the electron beam, and (c) their occurrence in otherwise clean fields on sparsely populated grids. As noted subsequently, the proportion of such nuclei with threadlike protrusions was found relatively constant by electron microscopy despite increasing ratio T/N as determined by phase microscopy. This also supports the view that the threadlike bodies seen with many nuclei were preformed and protruding structures rather than casual attachments as held by Lamb.

[1] Standard deviations are .18, .06 and .11 for the individual serial proportions of types O, D and T; maximum uncorrected *t* is less than 2, indicating no significant differences of the serial values from the overall means. Standard errors of overall means are .057, .019 and .035 for types *O*, *D*, and *T* respectively.

but 2.6 per cent and virtually 0 per cent observed above by phase microscopy. For the run with the *WB*, average incidences (and standard errors) for nuclei with threadlike or bodily protrusions were 54 (4.5) per cent and 18 (2.3) per cent. The proportions found in the serial specimens did not differ statistically from these overall averages in any instance and they were substantially unaltered in the final products (Table III). For the run with the *LM*, the corresponding incidences were 50 (3.5) per cent and 14 (1.9) per cent on the average for the mixes from the 10 extrusions. Again, these proportions for serial specimens did not differ statistically in any case from the overall averages, and they were not greatly altered in the final products (Table IV).

Similar results were obtained in two other runs with the *LM*. For these, nuclei were obtained by freezing and thawing cells in Gey's salt solution. The proportion of distorted nuclei determined by phase microscopy was low in both runs, averaging 6 per cent, while about half of the nuclei seen in the electron microscope were distorted. Again, however, these proportions were statistically constant throughout the treatment.

DISTORTED NUCLEI IN ATTEMPTS AT "OSMOTIC RUPTURE" OF THE CELLS

Rupture of cells by osmotic abuse was attempted in other work. Clean red cells prepared either in Gey's salt solution (pH *ca.* 7.6) or in M/4 sucrose (pH *ca.* 6.3) were mounted for phase or electron microscopy either as sedimented or after special exposure to water or to appropriate hypertonic media (M NaCl or 2M sucrose).

Phase examinations of wetmounts of the cells indicated their apparently good condition; some rounding and some apparently naked nuclei were

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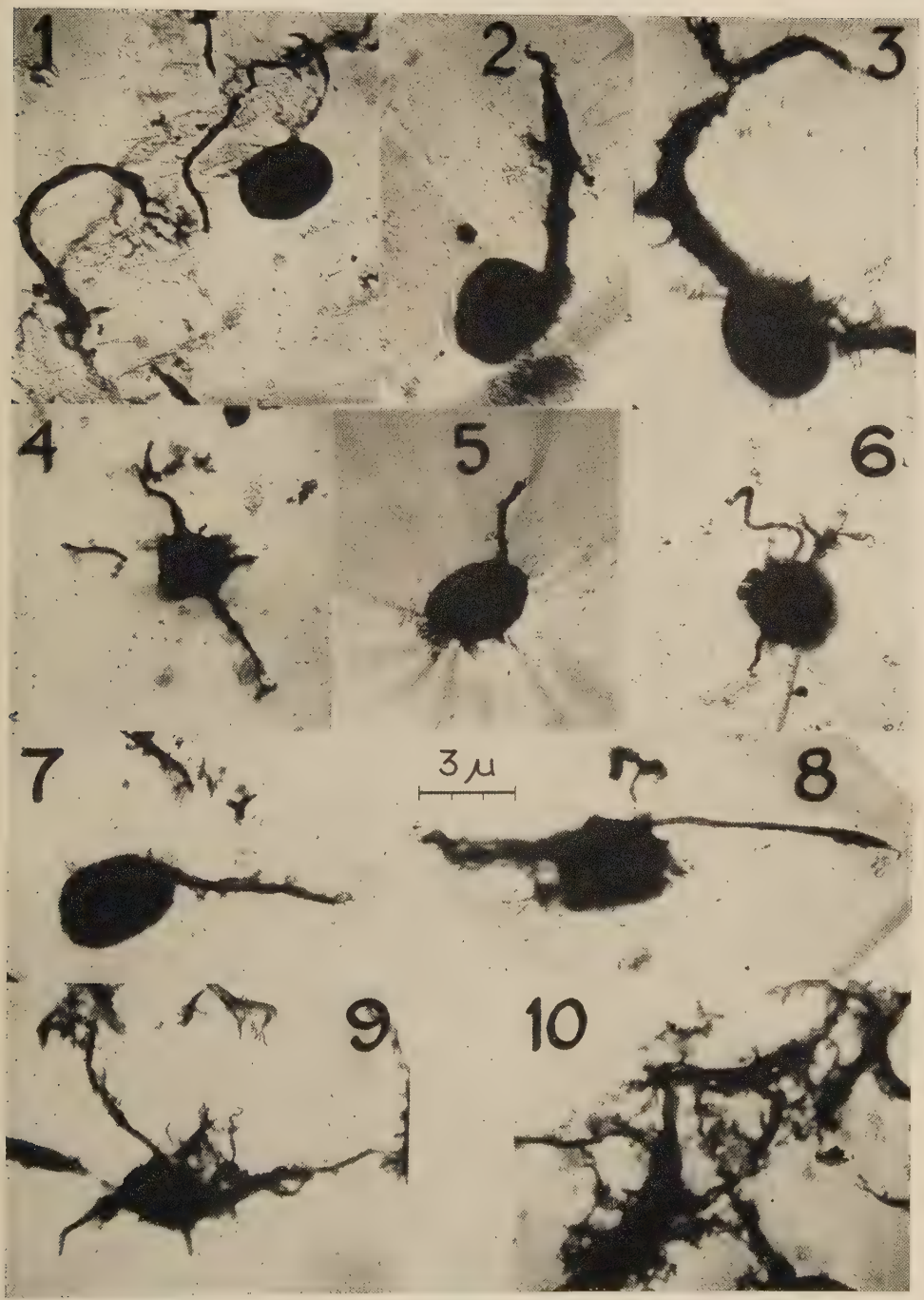
All illustrations are electron micrographs of material from chicken erythrocytes, made in a 50 kilovolt magnetic RCA microscope EMU-2B. Specimens were mounted on grids after the indicated treatment and were washed 10 times with glass-distilled water prior to air-drying. *AC/B* and *Os/B* indicate bulk-fixation of suspensions with equal volumes of acetocarmine or 2 per cent aqueous osmium tetroxide for at least 10 minutes. *WB* and *LM* indicate disruption of the material by Waring Blendor or by Logeman Mill.

Preparations of mechanically treated cells:

Figure 1 LM—Mix after 7 extrusions—AC/B
2 WB—Mix after 1 minute[1] —AC/B
3 LM—Mix after 2 extrusions—AC/B
4 LM—Mix after 8 extrusions—AC/B
5 WB—Mix after 1/2 minute —AC/B
6 LM—Mix after 3 extrusions—AC/B
Figure 7 LM—Mix after 6 extrusions—Os/B
8 WB—Mix after 1 minute —AC/B
9 LM—Final product, after 10 extrusions —Os/B
10 LM—Mix after 5 extrusions—AC/B

[1] Total time of full-speed treatment.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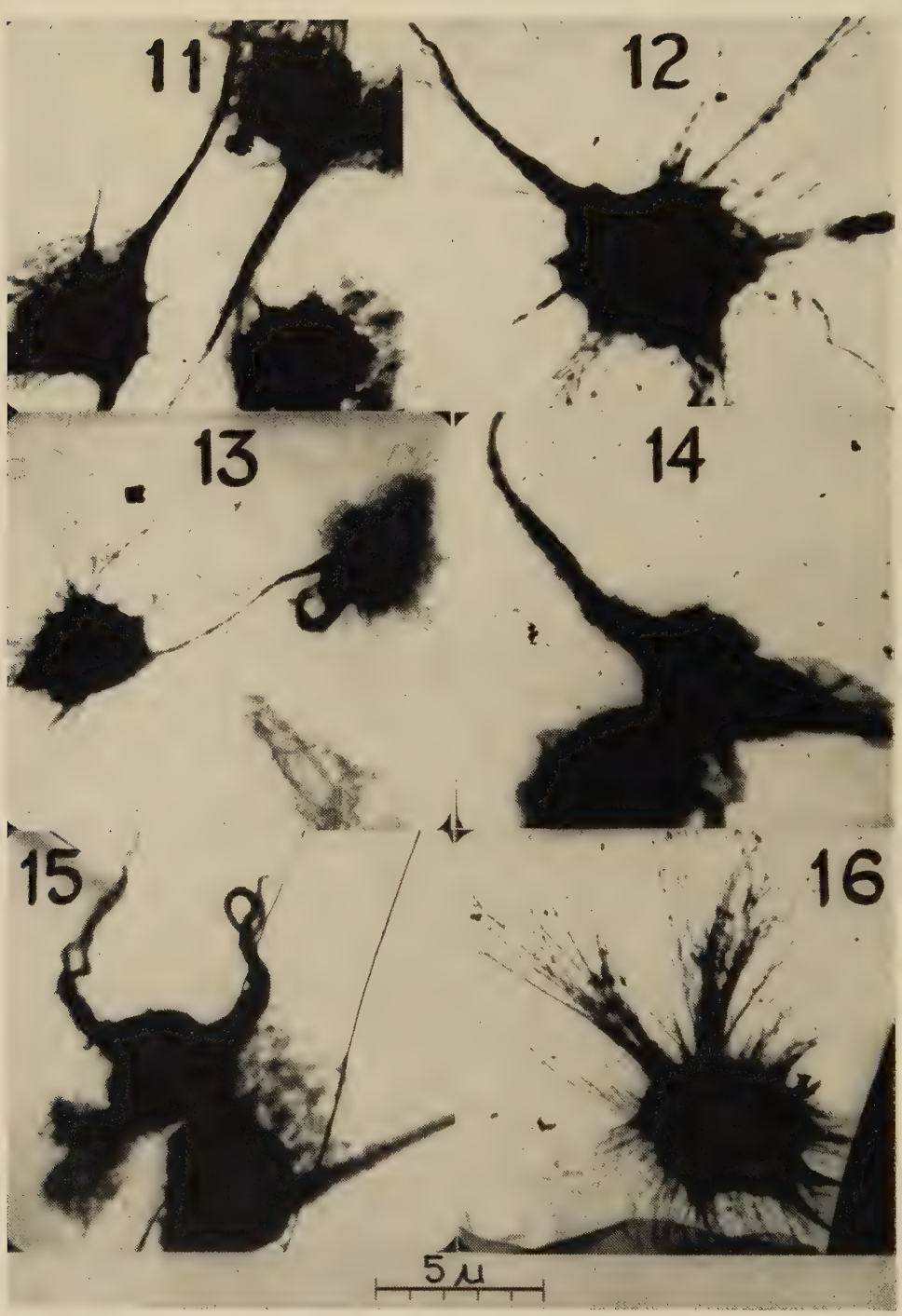
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seen in the sucrose material. For electron microscopy, which included about 350 individual micrographs, these cells were mounted unfixed or bulk-fixed with acetocarmine in three ways: in a standard fashion (allowing a drop of suspension to settle briefly on the grid, removing the excess and immediately washing 10 times with glass-distilled water and subsequently air-drying); by air-drying alone; or by air-drying followed by washing and redrying. Ruptured cells and free nuclei were regularly seen in all such preparations in the electron microscope. Cells normally predominated in these preparations but N/C was unity (30/29) in an acetocarmine-fixed preparation that was initially dried, then washed, and finally redried. The freed nuclei generally retained their round shapes. However, for unfixed sucrose material mounted in the standard fashion, characteristically tapering protrusions were regularly noted from splayed nuclei (Figs. 11 and 12). This finding was in contrast with typically round nuclei seen in the wetmounts of fresh material in the phase microscope and in similar material mounted for electron microscopy after fixation in acetocarmine. For hypotonic treatments in addition to the normal washing with water, standard mountings of these cells unfixed or after fixation with acetocarmine generally disclosed cells and nuclei with battered aspect and "drawn" appearance (e.g., Figs. 13, 14, and 15, the last including a protrusion apparently comprising two filaments). Exposure of cells from Gey's salt solution to M NaCl gave no free nuclei or distortions of interest. However, unfixed preparations from M/4 sucrose taken to 2M sucrose regularly showed free nuclei, some with extensive protrusions (Fig. 16).

Thus, distorted nuclei reminiscent of those described above were encountered in the electron microscope in unfixed standard mounts of untreated whole cells from M/4 sucrose; in unfixed or acetocarmine-fixed mounts of whole cells from M/4 sucrose of from Gey's salt solution that had been additionally exposed to water; and in unfixed mounts of whole cells from M/4 sucrose that had been exposed to 2M sucrose.

Preparations of whole cells untreated by WB or LM:

	Cells from	*Special Pretreatment*	*Fixation*
Figure 11	M/4 sucrose	None	Unfixed
12	M/4 sucrose	None	Unfixed
13	Gey's salt solution	Water soak, 1 minute	AC/B
14	Gey's salt solution	Water agitation by pipette, 1 minute	Unfixed
15	Gey's salt solution	Water agitaion by pipette, 1 minute	AC/B
16	M/4 surcose	2M sucrose soak, 1 minute	Unfixed

DISCUSSION

There is a striking disparity between our parallel observations of round nuclei in wetmounts by phase microscopy and of predominant distorted nuclei by electron microscopy. There is no doubt of the ready resolution in the light microscope of objects of the size of the massive protrusions and of many of the threadlike protrusions seen here only in the electron microscope. In the second experiments reported, distorted nuclei resembling those seen above resulted in preparations of whole cells exposed to water and to drying in standard mountings for electron microscopy. These features of the preparation of specimens for electron microscopy may thus underlie the difference noted.

This disparity is moreover just the qualitative difference between the reports by Mirsky and Ris (who by light microscopy saw very few distorted nuclei) and by Lamb (who by phase and electron microscopy saw many distorted nuclei). Lamb's paper includes no detailed documentation of the observations of fresh unfixed preparations in the phase microscope, and his illustrations of "nuclei undergoing disintegration in the Waring blender" (sic) are for fixed and stained material in light micrographs and for electron micrographs. Our localization of such a disparity in parallel preparations suggests its extraneous origin. The aptness of this argument is increased by the fact that Lamb used rather brief fixations (1 minute in acetic alcohol, 2 per cent osmium tetroxide, or Maximow's fixative) and multiple water-washings of the resuspended material, presumably immediately after such fixation.

Our detailed data do not support the hypothesis of Lamb, who pictured progressive massive distortion ("drawing") of nuclei to give random fragmentary threads. Distorted nuclei were indeed seen in our phase microscopic observations, but only with very small frequencies. In the electron microscope the proportions of distorted nuclei were high but statistically constant rather than progressive through the mechanical treatments. Distorted nuclei reminiscent of these were also seen in the electron microscopy of untreated whole cells and of whole cells subjected to osmotic abuse but to no mechanical treatment by *WB* or *LM*. Preparations of chromatin threads of the type seen here are surely not free of fragments of "drawn" nuclei. However, the above evidence argues against a central role of such distorted nuclei in the production of the characteristic chromatin threads in these preparations.

SUMMARY

Serial specimens were taken for phase and electron microscopic examinations during the isolation of chromatin threads from chicken erythrocytes with the Waring Blendor or the Logeman Mill. In fresh wetmounts, distorted or "drawn" nuclei were seen very infrequently; characteristic threads appeared progressively in the presence of round nuclei. In electron microscopy of parallel specimens, distorted nuclei were most common but the proportions of predominant nuclei with threadlike protrusions and of "drawn" nuclei were statistically constant and similar throughout the widely different

mechanical treatments. Grossly distorted nuclei with "drawn" aspect resulted when untreated whole cells were exposed to osmotic abuse and drying stresses incidental to preparations for electron microscopy. The origin of most of the distorted nuclei seen in the electron microscope thus appears extraneous. The results indicate collectively that "drawn" nuclei do not here play a central role in the generation of the characteristic chromatin threads, as was postulated by Lamb.

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III. LENGTH OF CHROMATIN THREADS AND SPEED OF THE WARING BLENDOR

Possible effects of the speed of the Waring Blendor (*WB*) on the lengths of "isolated chromosomes" will be considered here. Lamb has indicated (3) that shorter threads were obtained by *WB* than by a glass homogenizer except when the Blendor was operated at a "lower speed comparable with that of the homogenizer" (*ca.* 1000 RPM). In contrast, Ris and Mirsky (4) reported that the size and shape of the threads were unaffected by the time of treatment. No detailed data were given in these reports, and we have been unable to justify Lamb's indication by measurements from the few published micrographs.

An examination of this possible artificial role of the Waring Blendor has been made by comparing the lengths of threads isolated after disruption of the material at minimum and at maximum speeds.

TABLE I

Disruption of Chicken Erythrocytes by the Waring Blendor at Low Speeds[1].

Speed[2], RPM	N/C[3]	T/N[3]
4 000	0.26	0.0
5 000	[4]	[4]
6 000	.7	.5
7 500	1.4	.9

EXPERIMENTAL

A calibration of the speed of our Blendor was made at room temperature with water as a filling. Various voltages were applied to the *WB* motor by an autotransformer. Light was reflected from a sector of the hub on the shaft to a photocell, and the frequency of the resulting impulses was determined by comparison with an oscillator calibrated against a standard tuning fork. Using a standard filling of 50 ml of water, which just came to the horizontal blade, the resulting smooth speed-voltage curve ranged from 3000 RPM at 29.5 volts to 15700 RPM at 142 volts. Operation below 3000 RPM was erratic; the minimum voltage to start the motor was found to be 21 volts. Checks with overfilling with 100 ml showed less than 2 per cent effect on the speed at a given voltage; underfilling with 25 ml of water increased the speed 11 per cent at *ca.* 8000 RPM and 3 per cent at the top speed near 16000 RPM. These small effects suggested the adequacy of the drive. The data were therefore taken as indicative of the preparative speeds of a warmed *WB* motor used in the cold room.

Working with chicken erythrocytes generally as before (1) but employing heparinized blood and approximately neutral 0.14 M NaCl medium, determinations were made of the minimum speed of the *WB* to give a useful number of threads in a total time of treatment of 5 minutes (5 one-minute runs separated by cooling intervals). Counts of cells, nuclei, and threads in fresh wetmounts by phase microscope (Table I) indicated feasible production of characteristic threads only at speeds above *ca.* 6000 RPM. A minimal speed of 7500 RPM was selected.

Using a common suspension of cleaned cells at 20-fold dilution, runs were made at 7500 RPM for total times of 5 and 10 minutes, and at 15000 RPM for 2 1/2 and 5 minutes. Counts of the serial mixes characterized the ensuing disruptions (Table II). All of the final specimens were cleaned in parallel, by 4 washings of the threads at *ca.* 600 g for 10 minutes each. Mounts for electron microscopy were made in the

[1] From systematic counts of fresh wetmounts by phase microscope after 5 minutes of total treatment (increments of 1 minute separated by cooling periods).

[2] The minimum speed for stable operation was *ca.* 3000 RPM.

[3] *C* denotes number of intact cells, *N* the number of free intact nuclei, *T* the number of discrete free threads.

[4] Qualitative observations here indicated many persisting cells, free nuclei in abundance, and only occasional threads.

TABLE II

Disruption of Chicken Erythrocytes by the Waring Blendor in the Preparative Runs.[1]

Total time of treatment, minutes	Data for the shorter Runs		Data for the longer Runs	
	N/C	T/N	N/C	T/N
A. Runs at ca. 7 500 RPM:				
1	0.2	0.0	0.4	0.1
2	.5	.04	.8	.9
3	1.4	.6	.6	1.3
4	1.2	.6	1.3	1.0
5	1.0	3.4	1.0	2.2
6			.7	1.5
7			1.7	1.6
8			1.2	2.6
9			2.7	2.6
10			6.0	2.8
B. Runs at ca. 15 000 RPM:				
1	9	7	4	3.2
2	0/0	93/0	2.5	82
2 1/2	0/0	72	—	—
3			18/0	67[2]
4			13/0	90[2]
5			5/0	200

standard way after bulk-fixation with acetocarmine or with 2 per cent aqueous osmium tetroxide.

Representative electron-micrographs were recorded in 265 individual exposures. The lengths of all discrete threads appearing on these micrographs were determined with a "map measure", using projections. Approximate widths of the threads were also recorded. A few threads (numbering 19 and including the full range of lengths) were set aside as atypical; 496 measurements were retained. Grouped lengths, reported for brevity in Table III, are represented in the histograms of Figure 1. Some statistics derived from the *un*grouped data are given in Table IV.

DISCUSSION

Generally similar distributions of lengths of these chromatin threads are thus seen despite the twofold ranges in speed and in time of treatment.

[1] From systematic counts of fresh wetmounts by phase microscope. *C* denotes the number of intact cells, *N* the number of free intact nuclei, *T* the number of discrete free threads.

[2] Tangles of threads present, making counts unusually conservative.

TABLE III

Arrays of Lengths of Chromatin Threads Prepared with the Waring Blendor from Chicken Erythrocytes.

Length, micra	Speed 7 500 RPM		Speed 15 000 RPM	
	Total Time of Treatment		Total Time of Treatment	
	5 minutes	10 minutes	5 minutes	10 minutes
0– 1	1	0	7	0
1.1– 2	4	7	24	17
2.1– 3	10	4	9	21
3.1– 4	12	12	21	27
4.1– 5	5	10	18	32
5.1– 6	10	12	13	32
6.1– 7	10	6	12	25
7.1– 8	1	5	14	18
8.1– 9	5	1	8	15
9.1–10	1	3	6	12
10.1–11	1	3	9	5
11.1–12	1	2	5	1
12.1–13	0	1	4	0
13.1–14	1	0	1	0
14.1–15	0	0	1	4
15.1–16	0	0	1	2
16.1–17	0	0	0	2
17.1–18	0	0	0	0
18.1–19	0	0	0	1
19.1–20	0	0	0	1
Total number	62	66	153	215

When plotted cumulatively on probability paper these arrays though relatively limited appear reasonably normal, and they agree fairly well with the more extensive data previously obtained (1). The indicated valid difference of the extreme mean lengths is not considered striking in view of the nature of the data. The general and quantitative similarities are striking, however, and one may deduce that the twofold range of speed has no great effect on the lengths of the resulting chromatin threads. This indication holds for comparisons either at a constant time of treatment of 5 minutes or at two equal total numbers of revolutions of the impeller of the *WB*. These results thus do not support the indication of Lamb (3) that length varies inversely with speed of the *WB*; they agree with the absence of effect of time of treatment as reported by Ris and Mirsky (4).

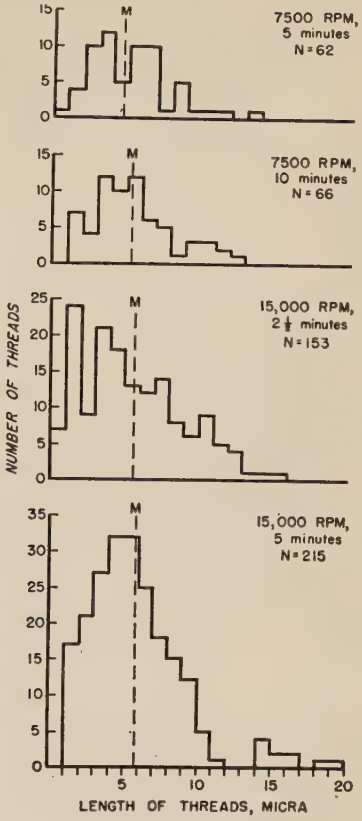

Fig. 1. Distributions of Lengths of Chromatin Threads Isolated from Chicken Erythrocytes with the Waring Blendor.

It might be argued that our minimum speed is above a value that assures a characteristic product from the *WB*. However, the results at lower speeds (Table I) suggest the threshold nature of the selected minimum speed, and the data of Table II emphasize the widely different disruptions attained. It might also be argued that centrifugal fractionation in the cleaning procedure has assured similar products. Supplementing our previous indication, however (in the last paragraph of Discussion in reference (1)), a subsequent run has verified the sedimentation of substantially all of the characteristic threadlike bodies under the conditions employed.

These findings are of increased importance because of the recently published view (2) attributing to the Blendor the helical structures indicated in such material, as seen by several workers. This general objection, based on the high turbulence and torsion obtaining in this apparatus, motivated our search for a system operating by essentially pure shear. The Logeman Mill was found to meet these requirements, and threads so obtained were found within limitations of method to be essentially similar to those obtained with the *WB* (1). The present results thus provide a second instance in which possible artificial effects of the method of preparation of isolated chromatin threads have not been verified.

TABLE IV

Statistics Derived from the Ungrouped Data for Chromatin Threads Prepared from Chicken Erythrocytes by Various Treatments with the Waring Blendor.

	Preparation at 7 500 RPM, Treatment		Preparation at 15 000 RPM, Treatment	
	5 minutes	10 minutes	5 minutes	10 minutes
Number of Threads	62	66	153	215
Mean Length, micra	4.6	5.4	5.6	5.8
Standard Deviation of Length, micra	2.6	2.7	3.5	3.3
Standard Error of Mean Length, micra	.33	.33	.28	.23
Median, micra	4.8	4.9	4.7	5.4
Estimated Mode, micra	3.5	4.5	—	5.0
Approximate Mean Width, micra	.3	.3	.3	.5
Estimates of Skewness:				
From Mean, Mode, and Standard Deviation	+.4	+.3	—	+.2
From First and Third Quartiles and Quartile Deviation	0	+.2	+.5	+.2

SUMMARY

Distributions of the lengths of chromatin threads isolated in salt solution from chicken erythrocytes with the Waring Blendor operated at a minimum speed and at the normal full speed were found very similar. These results do not support the indication (3) that the length of the threads is artificially influenced by the action of the Blendor.

The author is very grateful to a number of colleagues, especially to Dr. John J. Biesele for innumerable aids; to Mr. Louis Hildebrand for much preparative work and electron microscopy; to Theodor R. Marcus for making the many measurements of the threads; to Mones Berman for instrumental help in calibrating the Waring Blendor; and to Mark E. Gettner for servicing the electron microscope.

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A SIMPLE MICROSCOPE ATTACHMENT PERMITTING A CHANGE-OVER TO OPTICAL STAINING BY FLUORESCENCE AND POLARIZATION MICROSCOPY

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The equipment of standard fluorescence and polarization microscopy is usually sufficient in routine investigations, but not for detailed interpretations. Thus for example, certain structures may be discerned by fluorescence microscopy, but this does not imply that all structures of that kind are actually fluorescing. Moreover, it may be difficult to establish whether fluorescence occurs in a minute cytological structure or in some adjacent particle. It would be advantageous to reveal that in a single operation, thus avoiding time-consuming phase contrast microscopy or subsequent staining of the slide.

If a fluorescence or polarization microscope were provided with phase contrast equipment it might be possible to interpret the microscopical picture directly. Although it is possible to design a microscope adapted for a rapid change to phase contrast microscopy, optical staining procedures seem preferable.

A two-channel condenser is generally used for optical staining of microscopical preparations. Staining occurs when light reaching the preparation at different angles is diffracted (after having passed through two differently coloured filters in the two channels). Two cones of rays of different colours are produced. One forms a solid centre, the second a surrounding hollow. In this way large angles are formed between rays of different wavelengths. Since smaller angles seem preferable in this connection, the two-channel principle has been abandoned.

Owing to the chromatic aberration of ordinary condensers, small angles between light rays of different wavelengths are obtained at the level of the slide. In order to make use of these differences, the centre of the condenser was covered with a disc. This allows only a narrow ring from the periphery to reach the aperture of the objective. Hollow cones of different colours with their common tops reaching the preparation on the slide may then be said

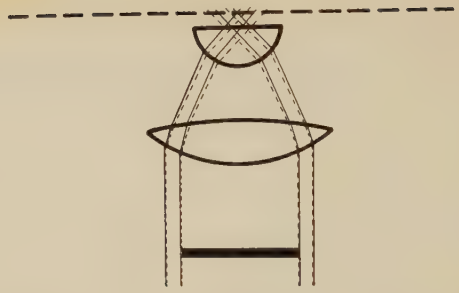

Fig. 1. Principles of illumination.
The condenser is screened off by a metal disc. Mixed light of two different wavelengths passes the margin of the disc. Because of the chromatic aberration of the condenser the rays reach the level of the preparation at different angles, as indicated by the dotted line.

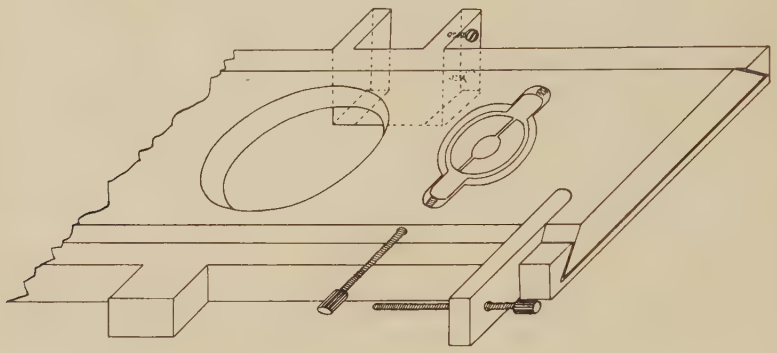

Fig. 2. Outline of the attachment. The slide is fitted in a holder provided with a stop and may be moved to the desired lateral positions after adjustment of the screws. When the central aperture is used the slide is moved to the position seen in the figure. A rod in the lateral apertures is adjusted by a screw and a spring. When the rods were cut a frame of the metal was left to make the mounting of the metal disc easy.

to constitute the system of illumination (See Fig. 1). For successful optical staining the filters must be appropriately selected. If one should choose to stain with blue and red light, suitable filters are available which transmit these wavelengths and absorb most of the remainder of the visible spectrum. An ordinary filament bulb might thus be combined with the filters BG1 and BG25 (Schott, Jena). It has been found desirable, however, to add a third filter (e.g. euphos glass) in order to reduce the intensity of the blue light. If a strong and efficient mercury lamp is used, the choice of filters is easier, since several ultra violet filters transmit blue and red light to a sufficient extent. The surplus of blue light must then be reduced by a red-transmitting filter which does not absorb all the blue light. Owing to their inferior

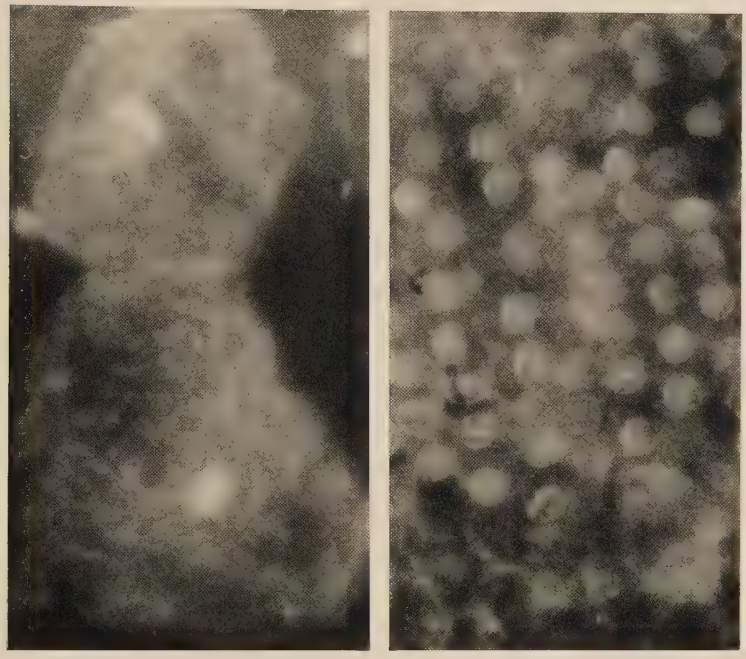

Fig. 3. To the left epithelial cells scraped from the buccal mucosa; to the right the primordium of the lens fibre in a stripped and unrolled capsule of the eye lens. When these preparations were viewed, the nuclei and also some smaller refractile particles appeared red against the cytoplasm. The refractile parts are lighter in the photomicrographs, but the monochrome reproduction is not accurate.

quality, some red filters intended for photographic use possess such properties.

For the purpose of optical staining the condenser attachment may be more or less elaborate. The mechanical equipment of most institutes will, however, probably be sufficient. The attachment used by this writer for fluorescence microscopy is constructed in the following way. (See Fig. 2.) A light metal slide is mounted on a holder which is screwed to the substage condenser. This movable slide is provided with three holes containing filters. An additional filter transmitting ultra-violet, blue and red light is placed in the aperture of the holder. The middle hole of the slide contains an ultra violet filter of adequate quality. If this aperture is used, the system is ready for fluorescence microscopy. The lateral holes contain a red filter and a metal disc, each fitting different objectives. In the lateral apertures there is also a metal rod which is pressed against a spring by a screw. The plate mounted on the rod may thus be moved perpendicularly

to the long axis of the slide. To facilitate adjustment in the other direction, the slide movements are arrested by adjustable screws (see Fig. 2). It was found convenient to use commercially available screws with threaded collars, which were fixed in drilled holes in the slide. When the centering of the plate has been accomplished (by aid of an auxiliary microscope), a blue and a red annular area are observed around the margin of the plate. These areas partly overlap and a pink or purple colour occurs in the common zone. Optical staining and fluorescence microscopy can take place alternately without repeated adjustment.

Though it is possible to combine the described attachment with several condensers, the result is not always entirely satisfactory. It may be improved by adding an auxiliary lens to the condenser. This may be selected through preliminary trials with lenses used in common ophthalmological refraction tests. A quartz condenser used by this writer was provided with a specially manufactured quartz lens of +2.5 dioptres which was mounted in the slide holder and kept in position during the change-over between fluorescence microscopy and optical staining.

Optical staining is especially convenient when examining single cells, but thin tissue slices may also be investigated. The photomicrograph in Fig. 3 illustrates to some extent the results obtained by the method (although only reproduced in black and white).

SUMMARY

A microscope attachment is described which makes optical staining possible without using a two-channel condenser. The attachment can be combined with different condensers and can be instantly moved allowing an alternating use of the microscope. This has been found useful in fluorescence microscopy, and may also be recommended in other connections.

ELECTRON MICROSCOPE STUDY OF MOUSE MAMMARY CARCINOMA TISSUE[1]

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The particulate bodies, supposedly associated with the induction of mammary carcinoma in certain strains of mice, have been studied with the electron microscope by several workers. For technical reasons, the specimens used have been fractionated preparations from mammary carcinoma tissue and milk of susceptible mice. Published reports show considerable variation in the estimated size of these particles (11, 14). This apparent variation in size could be due to alteration of the particles by the preparation procedures. It is also possible that the particles may have been confused in some cases with the particulate elements ordinarily present in normal cells. Porter and Thompson (15) chose an approach less subject to criticism by employing epithelial cells cultured from mammary carcinoma tissue. They interpreted the particulate bodies as being closely associated with the epithelial cells and suggested they were probably of exogenous origin and possibly a virus. The portions which they observed were the thin parts of flattened cells.

It is desirable, if possible, to obtain a true picture of all portions of the cancer cells in the various regions of the tumor tissue. Such findings would give important information regarding the particles in their natural sites and their relationship with known intracellular structures. The thin sectioning technique for the electron microscope has made it possible to obtain clear images of cross sections of cancer cells in the carcinoma tissue. The results and observations reported in this paper were obtained by use of this technique.

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[2] Public Health Service Special Research Fellow of the National Cancer Institute.

[3] Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions of the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

MATERIAL AND METHOD

For the present study a transplantable mammary carcinoma of a CBA mouse was used. The tumor had originally developed spontaneously in an adult female mouse of CBA strain in our laboratory and had been passed through four generations by successive subcutaneous transplantation. The tumor was histologically a typical adenocarcinoma, grew rapidly, metastasized widely, and showed a high degree of transplantability. Specimens were taken from the nodules in the growing periphery of a tumor which had been transplanted three to four weeks previously. For electron microscopy a piece 3 mm in diameter was removed, fixed in 10 per cent formaldehyde and then 1 per cent osmic acid solution. The tissue was embedded in N. butyl methacrylate (13) and sectioned with a modified Spencer 820 microtome (3). The adjoining part of the tumor tissue was taken for light microscopy.

The characteristic features of cancer tissue were first confirmed in the routine microscopic preparation. Referring to these findings, a region with typical adenocarcinomatous structures was located at low power with the electron microscope. Identification was accomplished by finding the pseudorosette formation or an alveolar cell-mass with interstitial lining. After positive identification of carcinoma cells was made at low power, the magnification was increased.

For comparison, a growing nodular part of the tumor tissue was homogenized with a glass homogenizer and the material was suspended in 0.5 M sucrose solution. This suspension was centrifuged twice at 600 times gravity for ten minutes and twice at 4 500 times gravity for thirty minutes in a Spinco ultracentrifuge maintained at —8° C. The preparations were kept in an ice bath when not in the centrifuge. After each centrifugation the supernatant layer was carried over to the next centrifugation. Specimens for the electron microscope were made from the last supernatant fluid obtained after 4 500 times gravity.

RESULTS

Our findings concerning the particulate bodies were similar but not identical with those described by other workers who observed fractionated preparations (11, 14) or cultured epithelial cells (15).

The particulate bodies, which could be easily distinguished from the normal intracellular structural bodies, were found in the malignant cells, but not in the endothelial cells, histiocytes, fibroblasts, or blood cells. However, a small number of particulate bodies were observed outside of the cell in a small lumen of the adenomatous structure. They were located close to a carcinoma cell which contained an abundance of particulate bodies and looked as if they had been liberated from the broken, free end of the carcinoma cell.

The particulate bodies were spherical and appeared undistorted unless they were densely packed in a mass. The outside boundary of the particles

was extremely well defined, usually limited by a dark circular line. The overall dimension was readily measurable and fairly constant, ranging from 140 to 180 millimicrons. (Figs. 1 and 2.)

The internal structures of the particles were somewhat like those of papilloma virus reported by Sharp and others (16). Each particulate body, with a few exceptions, had a single region of contrast, higher than that of the remainder of the body. The boundary of the dense region was not well limited but faded irregularly into the surrounding pale zone. The size of the internal dense region was, therefore, not accurately measurable. Its dimensions were not constant, ranging from small to extremely large size almost filling the entire space of the particulate body. (Figs. 1 and 2.) Our observations regarding the outer boundary and the boundary of the internal dense region were very different from those reported by Porter and Thompson (15). They have observed the diameter of the central, dense core as being fairly constant from particle to particle, while the overall dimension was more variable, due to the fact that it was often poorly defined and not accurately measurable. They have suggested that the pale portion of the particle, presumably less rigid than the core, could have been readily distorted.

In our preparations, the dense region was not always found in the center of the body, but usually situated more or less eccentrically. (Figs. 1 and 2.) This eccentric location suggests that the region of higher contrast was not an artefact produced by incomplete drying of the central zone during desiccation. Particulate bodies without a dense region were occasionally observed. (Figs. 1 and 2.) Such bodies are probably similar to the ghost forms of vaccinia virus described by Green *et al.* (12). It must be assumed that either the cross section fell on the level which excluded the part with the dense region or else the substance comprising the dark region was not present in a sufficient amount.

Occasionally two small dense regions, mostly asymmetrical in size and shape, were found in the larger of the particulate bodies. However, no image was observed suggesting a direct division of the particle. The rosette arrangement of dense granules, such as the nebular system described by Porter and Thompson (15) and Gessler *et al.* (10), was not observed. However, extremely large bodies with several small and large dense regions, irregularly situated, were found. This formation was practically indistinguishable from a compact mass of several particulate bodies.

The number of particulate bodies varied considerably in the different carcinoma cells of the same tissue. Variation from a few particles to a

large number almost filling an entire cell was observed. The large malignant cell usually contained a greater number of particulate bodies than the smaller. In some cross sections of carcinoma cells the particulate bodies were not found at all. They appeared to be restricted to the tumor cells of certain parts of the tumor tissue.

The bodies were observed lying scattered singularly in the cytoplasm and also arranged in pairs or in compact groups. There appeared to be no tendency to line up in rows. There was no characteristic localization in the cytoplasm suggesting any relationship with the cellular structures. (Figs. 1 and 3.)

The particulate bodies were observed within the nucleus of the malignant cell. This observation was made on a thin cross section of the nucleus or an area bordered by the thick nuclear membrane, instead of the whole cell. These particles, therefore, were actually within the nuclear structure (Figs. 3 and 4). Porter and Thompson (15) reported that a few micrographs had shown the particles in the area of the nucleus. They were inclined to believe that the particles were between the cell and the nuclear membrane. However, no decisive interpretation could be given because they observed the entire flattened cell. In this case, it would be difficult to ascertain whether the particles were actually in the cytoplasm or merely superimposed on the cell-strand. In our preparations the cross section of the nucleus was sometimes filled with the particulate bodies, especially in the tumor cells with abundant particles in the cytoplasm. The particles seem to group in large or small round masses each of which was occasionally distinctly encircled by a dark line. It appeared that the particles had developed, forming a secondary structure within the nucleus and distended the boundary of the structure as they increased in number (Figs. 3 and 4).

In the supernatant after centrifugation at 4 500 times gravity, particles with dense internal regions similar to those described above were found, although comparatively few in number. Besides these, there were many dark, smaller particles comparable in shape and size to the dense regions seen in the particulate bodies.

DISCUSSION

The tumor tissue employed in this study was from a transplantable mammary carcinoma which originally developed spontaneously in a colony of inbred CBA mice. The tumor was similar in every respect to those known

to be associated with the milk agent or inciting factor. In this study no attempt has been made to identify such a factor.

Barnum *et al.* (4) have reported that the tumor tissue derived from a spontaneous mammary tumor arising in an A strain mouse and carried for two generations seemed to be a relatively poor and unreliable source of the milk agent. Bittner (5), however, readily demonstrated the milk agent in transplanted mammary tumor tissue carried for ten generations in C 57 mice. The transplantable mammary tumor used in this study was only of the fourth passage. However, it seems probable that the milk agent would be demonstrable in this tumor tissue.

Bittner (6, 7) reviewed the characteristics of the milk factor as compared with those of known infectious agents or viruses. Many investigators (10, 11, 14) consider the spherical bodies demonstrated by electron microscopy to be the milk factor. However, there is no direct evidence that such is the case. Porter and Thompson (15) regarded the uniform morphology of the particles, their association in closely packed clumps and their irregular occurrence in the tumor cells, as being especially significant features distinguishing them from normal components of the cell, and made it seem probable that the particles were of extraneous origin and possibly a virus.

In the present investigation the particulate bodies of characteristic shape and size were observed in the tumor tissue. Although the observations were limited to carcinoma tissue, cells other than the tumor cells and blood cells were also found in the preparations. It seems quite significant that the particulate bodies were detected exclusively in the malignant cells and not in any of the other cells of the blood or tissue. Bittner (6), on the other hand, has cited various normal tissues, including whole blood and serum, from which the tumor-inducing milk agent might be recovered by fractionation and extraction. Gardner (9) suggests that the occurrence of the agent in milk might be due to the presence of the agent in lymphoid elements or other formed elements such as macrophages. Since normal cells, reported to contain the causative agent, do not appear to contain the particulate bodies, careful consideration and investigation should be required before accepting the tumor causing milk factor and the electron microscope particulate bodies as being one and the same.

The present observation revealed some tumor cells which did not appear to contain particulate bodies. If the milk agent is responsible for maintaining malignancy and is carried over to the daughter cells, all the tumor cells should contain the agent. The possibility that the cross section technique excluded the portion containing the particulate bodies was improbable,

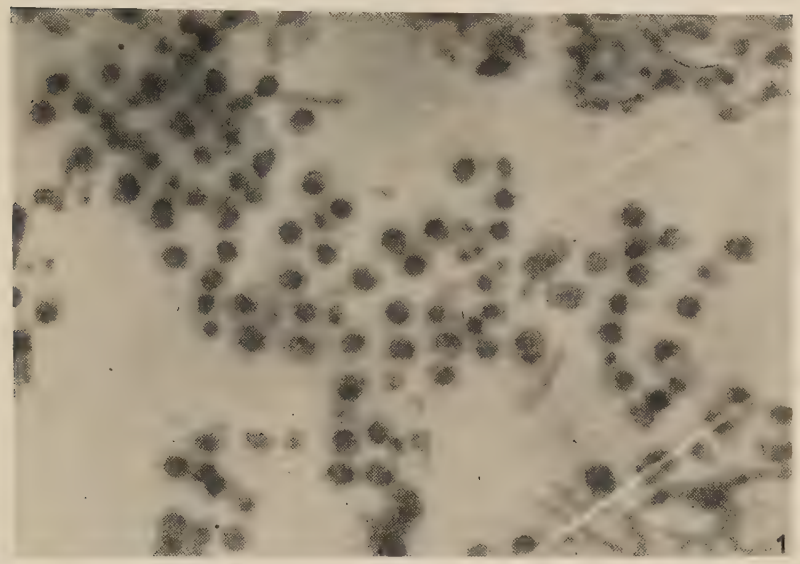
because many tumor cells without the particulate bodies were found grouped together and also scattered singularly throughout a section. It is therefore understood that even the tumor cells without the particulate bodies could remain malignant and produce malignant daughter cells. This being the case, the particulate bodies cannot act in the capacity of the continuing cause of the malignancy.

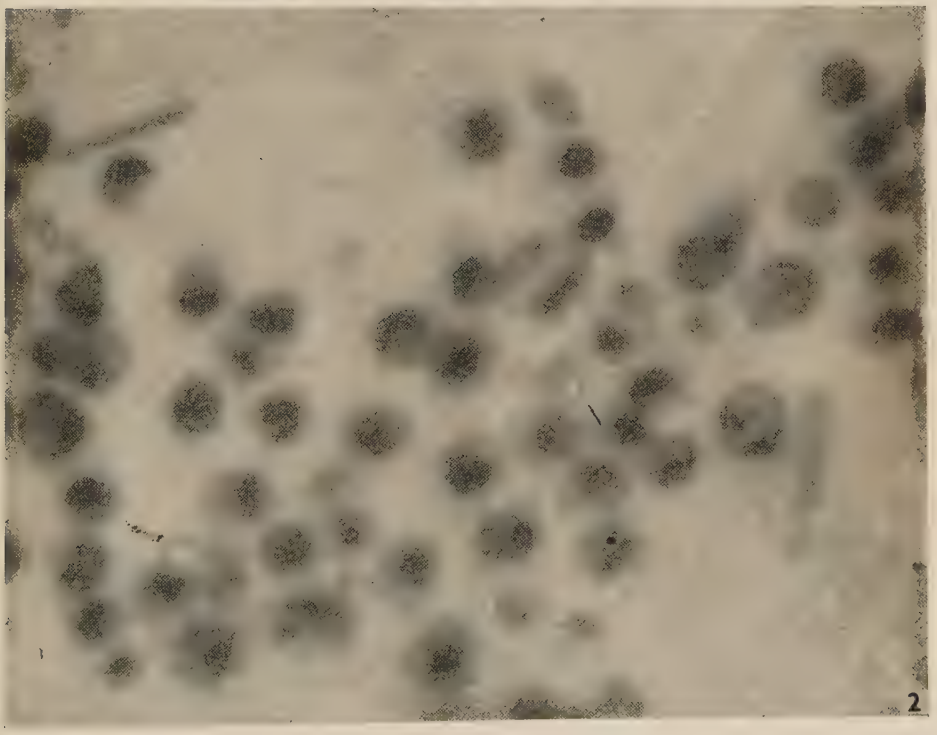
Andervont (2) has assumed that failure of tumors to occur in mice is associated with the disappearance or inactivation of the agent after successive generations of foster nursing. This suggests that the active agent is responsible for provoking the initial development of the tumor cells. It is conceivable that the particulate bodies observed might act in this capacity. Andervont (1) has also warned that with the mammary carcinoma, interpretations should not be too dependent upon experiences with so-called virus induced tumors which are produced rapidly with filtrates at the site of administration. Some experimental observations suggest that the agent may be of endogenous origin (8). Since these bodies seem to increase in number as the tumor cell became larger or older, they could be an endogenous product of the tumor cells at a certain stage of the cell development.

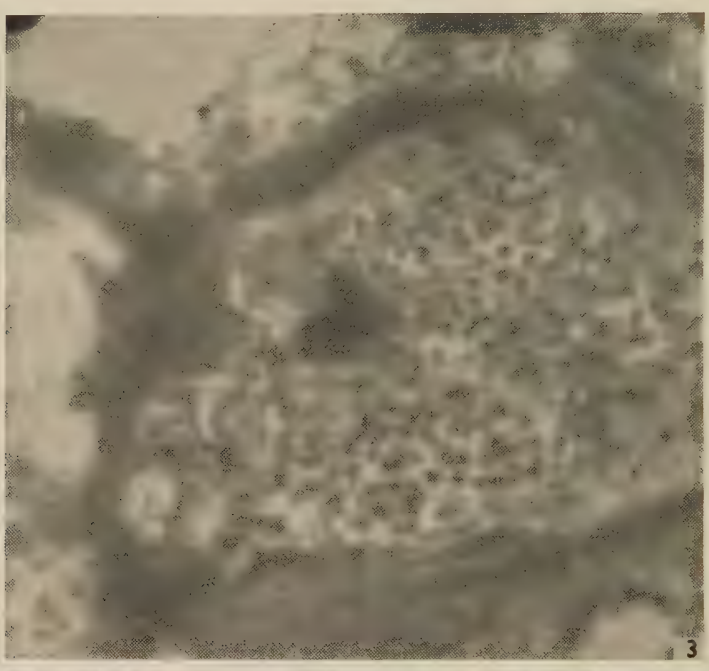
Regardless of the endogenous or exogenous origin it appears likely that particulate bodies increase within the tumor cells. The variation in their numbers and the arrangement of their grouping within the tumor cells support this assumption. Moreover, the large number of the particulate bodies and their secondary group-formations in the nucleus suggest that the nucleus may be an essential site of multiplication. However, there was no evidence to suggest a direct division as a mode of multiplication.

Passey and Dmochowski (14) stated that the supernatant of tumor extracts, obtained by centrifugation for one to two hours at 50 000 to 60 000 times gravity, contained large numbers of the typical particles. The most frequent diameters were 30, 20, and 35 millimicrons, in that order, but there were other particles present, in sizes ranging up to 120 millimicrons. The smaller particles may be comparable to the dense regions in the particulate bodies found in tissue sections. The large ones may correspond to the intact particulate bodies. Our homogenized and centrifuged preparations also showed many dense small particles and larger bodies with a dense region. The discrepancy in the diameter of particles reported by Passey and Dmochowski (14) is very likely due to the preparation procedure.

Fig. 1. Particulate bodies in mammary carcinoma tissue. Magnification 22 500 ×.
Fig. 2. Enlarged central portion of Fig. 1. Magnification 40 000 ×.



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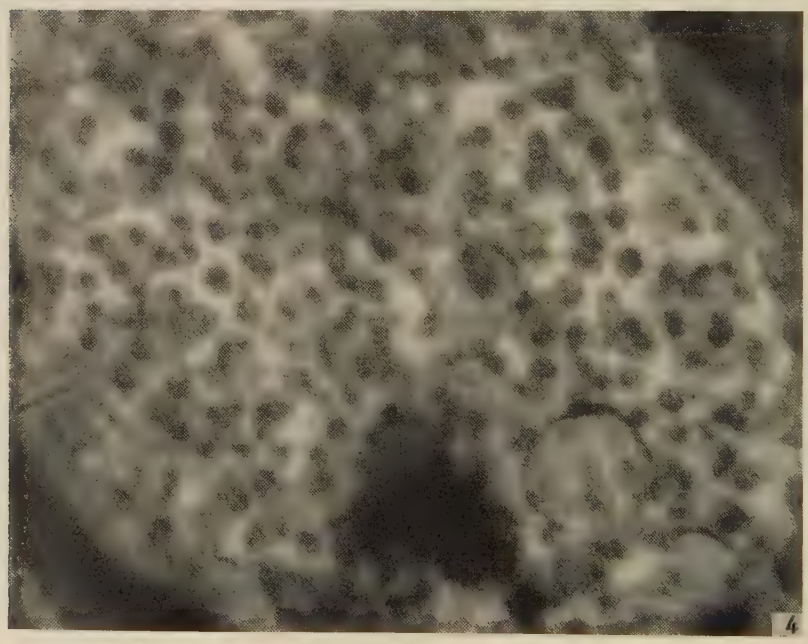
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Passey and Dmochowski also emphasized that their cell extracts, when tested in a high-cancer stain, were biologically active. This however, does not necessarily mean that the particles represent the active agent.

The dense region in the particulate bodies is understood to contain high electron absorbing material. This material could be nucleic acids, since nucleic acids carry atoms of high atomic number, such as phosphorus. This region might be essential for specific activity, if the activity is attributed to the particles. In such a case the dense region could grow in size by further deposition.

SUMMARY

Thin sections of mouse mammary carcinoma tissue were studied by electron microscopy. The features of the particulate bodies found in the tumor cells are described and compared with findings reported by other workers. The nature of the particulate bodies is discussed and the identification of the particulate bodies as being identical to the carcinogenic milk factor is questioned. Intranuclear development of the particulate bodies is suggested.

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Fig. 3. Intranuclear particulate bodies in mammary carcinoma tissue. Magnification 15 000 ×.
Fig. 4. Enlarged central portion of Fig. 2 A. Magnification 22,500 ×.

VIRUS FORMATION IN CHLOROPLASTS

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The idea that the chloroplasts were the sites of virus formation in the cell is not new. Kausche and Ruska (7) assumed formation of tobacco mosaic virus in the green plastids and published an electron micrograph showing filamentous particles protruding from a chloroplast fragment; they also assumed a definite relationship between the protein component of the chloroplast and the "virus protein." However, this work was done at a time when the techniques of electron microscopy were not very advanced, and one cannot be certain that the micrograph published actually shows rods of tobacco mosaic virus.

Black, Morgan and Wyckoff (3) published electron micrographs of sections of leaves infected with tobacco mosaic virus. Black's own view of the subject is of interest. He says: ". . . the electron micrography . . . shows apparent association of the virus particles with chloroplasts. However, virus is present in the cytoplasm and so far these micrographs cannot be considered to have proved what the site of virus multiplication is in this case" (2).

Many less direct observations have been made which may suggest virus multiplication in chloroplasts, for example by Kausche on the activity of preparations of chloroplasts from virus infected leaves (7) and by van Slogteren and co-workers on serological reactions (2).

Many observations on symptoms and on the influence of external factors on virus infection and on virus multiplication, which have been summarized in textbooks such as those by Bawden and Smith, might be better understood, if the phenomena could be related to the chloroplasts.

Chloroplasts are well differentiated from the rest of the cell and can be isolated (5, 11). Their structure (10), chemical composition (5, 11, 12, 15), and enzymatic activities (4) have been studied. If the propagation of virus is going on in the plastids even if there is an interaction with the surrounding protoplasm, it offers a suitable line of investigations on the physiology of virus formation.

In this paper, evidence will be given suggesting that the filamentous structures occurring in leaves infected with beet yellows virus are formed in chloroplasts. Some results will also be added which support the suggestions that the rods of tobacco mosaic virus originate from the plastids.

MATERIAL AND METHODS

Beet yellows virus. In an earlier report (9) filaments found in the sap of leaves infected with beet yellows virus and which could not be found in sap from healthy leaves were described. The precipitate of infected sap with its specific antiserum was shown to consist largely of the same sort of filaments. As the filaments were found often protruding from fragments of chloroplasts, it was suggested that they had been formed in the chloroplasts.

The evidence that the filamentous particles in leaves infected with beet yellows virus constitute the virus itself, is not as strong as the corresponding evidence for the rods of tobacco mosaic virus, for example, although there are obvious indications (9, 13). Whatever the nature of these threadlike structures, the results presented here have a bearing on the problem of virus formation and may contribute to our knowledge of beet yellows virus.

Chenopodium foliosum was used as a host plant for beet yellows virus; plants were infected with yellows obtained from a Swedish sugar-beet crop, using the aphid *Myzus persicae* (Sulzer) as a vector. The plants grew well under glass house conditions, and infected sap was found to contain more threadlike particles than comparable sugar-beet sap. Two to three weeks after infection the leaves showed strong vein yellowing followed by necrosis. When showing clear symptoms they were ground in a mortar, the sap was pressed through muslin, and tissue debris, free nuclei, starch grains, and chloroplasts were centrifuged down (half an hour at 700 g). The chloroplasts formed the upper layer of the sediment. Small samples of this layer were dispersed in distilled water and serial 5-fold dilutions were immediately prepared for electron microscopy. The preparations were shadow-cast with gold-manganin 1 : 1. Also the supernatant solution obtained after the centrifugation was studied.

RESULTS

Electron microscopy of sections has shown that the chloroplasts of the leaves are built up of stacks of very thin disks embedded in a stroma (10). After the treatment with distilled water, practically all chloroplasts are disrupted. The disks and the more or less rounded cavities where disks have been torn out from the stroma (Fig. 1) are typical of the chloroplast fragments. These fragments may thus be identified under the electron microscope.

The supernatant fluid of centrifuged sap from leaves infected with beet

yellows virus contained many chloroplast fragments of the type shown in Fig. 1. The threads protrude from the chloroplast fragment in such a way that they give the impression of having been formed there.

Chloroplast fragments from the sediment with disks still in position also occur in association with filaments. Now and then, *Chenopodium*-chloroplasts were found with a very large number of filaments (Fig. 2).

The threadlike structures adhere to chloroplast fragments more firmly than for example the rods of tobacco mosaic and potato X virus and this may be the main reason why attempts to purify the virus have not been successful so far (8). Thus it was considered worth while studying this material to find out in which part of the chloroplast the filaments are being formed. Many electron micrographs of which one is published here (Fig. 1), indicate that the propagation occurs in the stroma of the plastid. However, in preparations from supernatants, one often finds free chloroplast disks associated with filaments. This association is more common than might be expected from a random distribution of the two kinds of structures. Since many free disks and filaments are also found, the combination between them does not give the impression of being a result of adsorption. After thorough examination of many preparations under the electron microscope, some very thin chloroplast fragments were found with filaments and disks preserved in a seemingly original state of arrangement. Such pictures are shown in Figs. 3, 4, and 5. The findings indicate that the borders between stroma and disks are the sites of formation of the filaments. However, isolated disks may have some stroma attached to them, and it is thus very difficult to decide whether the filaments grow out from the stroma near the disks or from the disks themselves. This question will be of great interest if it can be shown in the future that disks and stroma have a different chemical composition.

Tobacco mosaic virus. It seemed of interest to try to reproduce the results obtained for tissue infected with beet yellows virus on other more or less filamentous structures commonly considered as virus. Tobacco mosaic and potato X viruses were first chosen since they are both known to occur in high concentration in crude sap of infected plants.

Turkish tobacco (*Nicotiana tabacum*, var. *bashi bagli gestielt*) infected with tobacco mosaic virus (strain *T 23* which was isolated in the State Plant Protection Institute in Stockholm in 1941 from a tomato plant) was used. Leaves showing symptoms of systemic infection were treated in the same way as the leaves infected with beet yellows virus.

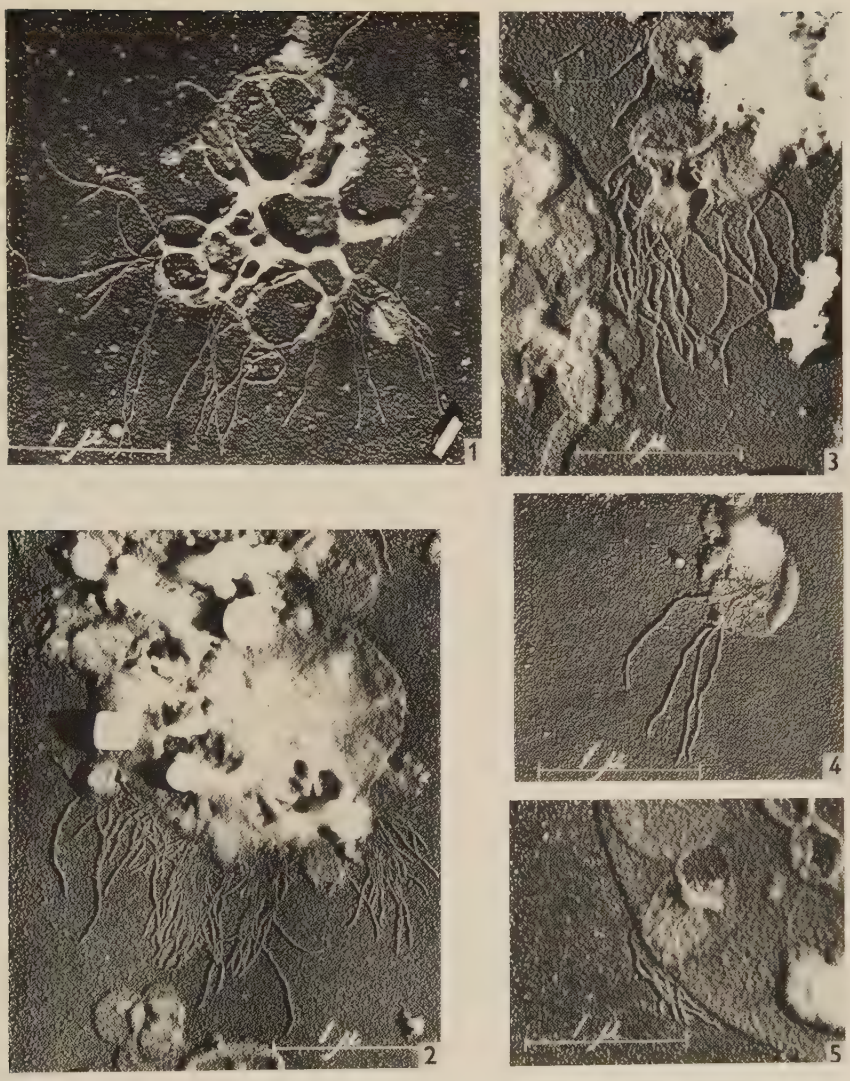


Fig. 1. Fragment of a chloroplast of *Beta sacharifera* infected with beet yellows virus (Leyon, *Arkiv Kemi*, **3**, 105 (1951)).
Fig. 2–5. Fragment of a chloroplast of *Chenopodium foliosum* infected with beet yellows virus.

Rods and fragments of chloroplasts were found in large numbers in the preparations. There was no general concentration of rods on or near the fragments which would have been expected, had there been any tendency for them to be adsorbed on to the fragments. Some of the chloroplast frag-

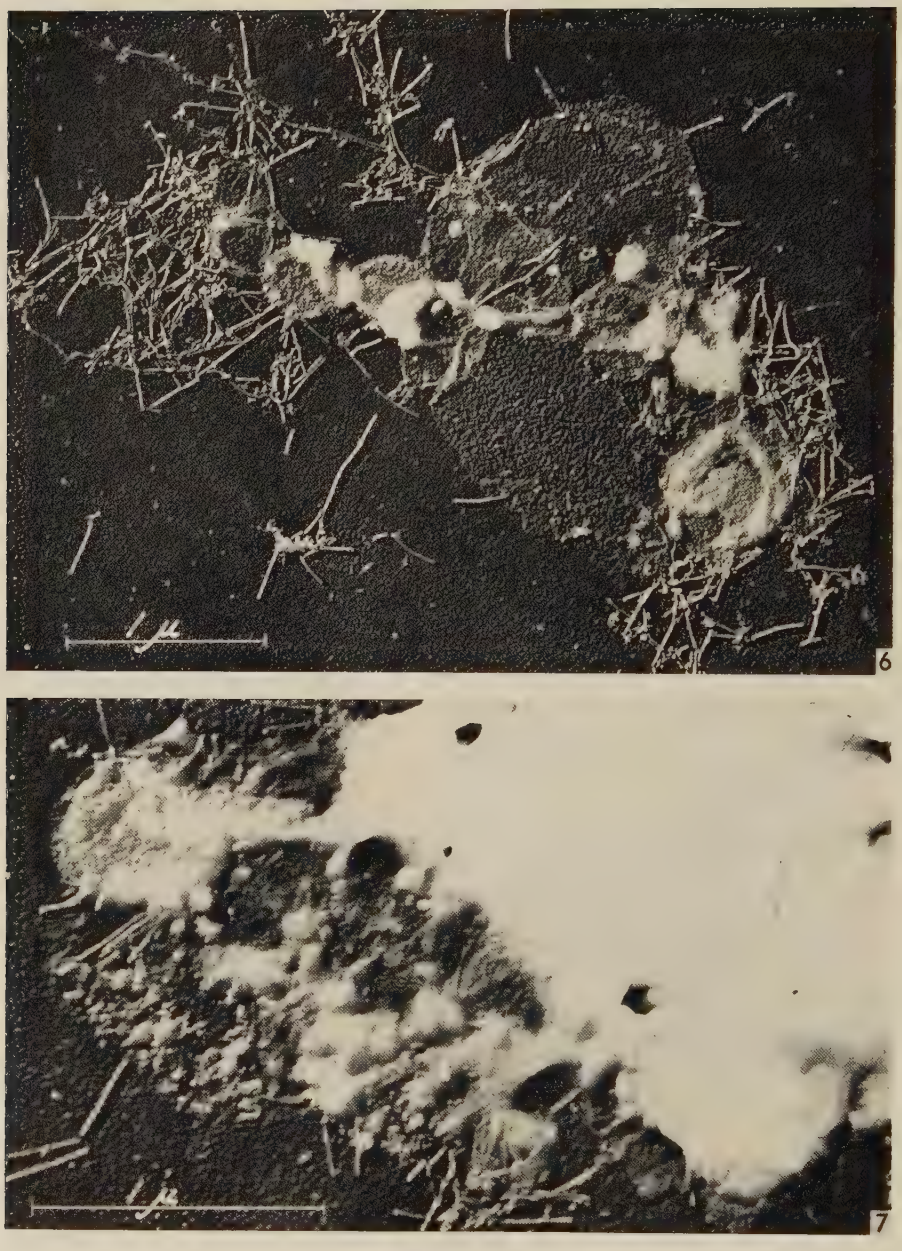
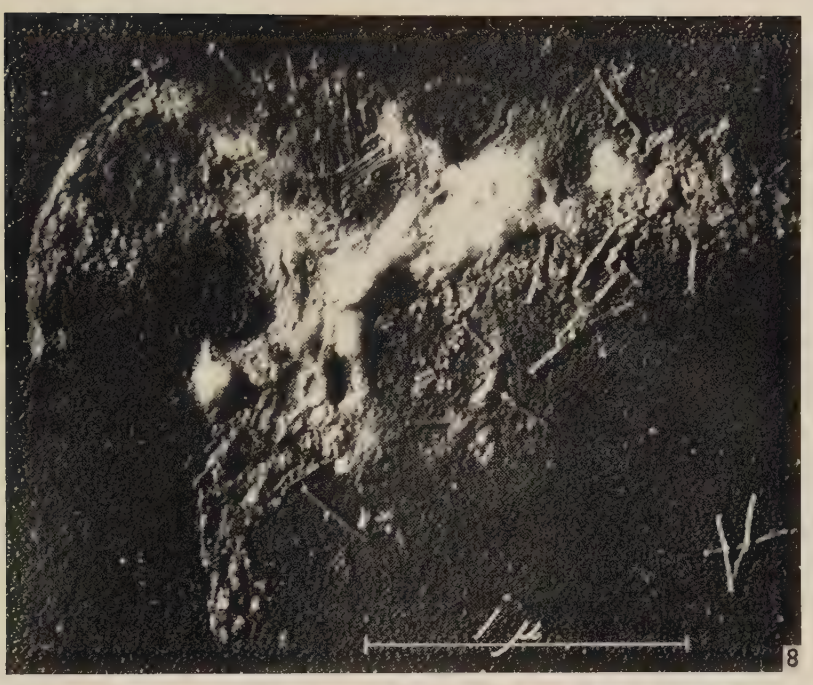
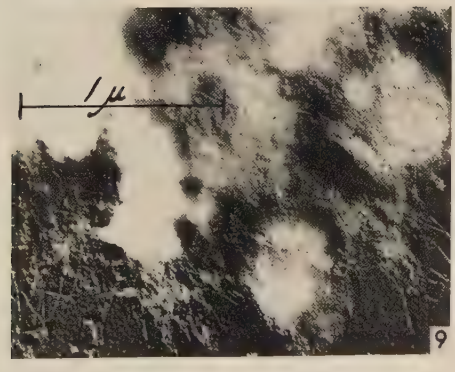


Fig. 6–8. Fragments of a chloroplast of *Nicotiana tabacum* infected with tobacco mosaic virus.





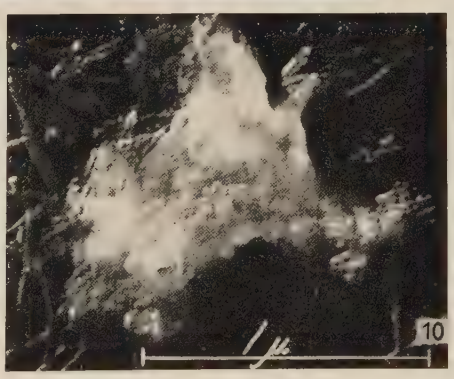


Fig. 9. Fragment of a chloroplast of *Nicotiana tabacum* infected with potato X virus.
Fig. 10. Fragment from tissue of *Nicotiana tabacum* infected with tobacco mosaic virus.

ments were, however, surrounded by large amounts of rods and some cell fragments similar to chloroplast fragments contained rods embedded in their plasma. (Figs. 6, 7, and 8.) In fragments containing much virus, the particles often occurred in bundles of parallel rods which was not the

case outside the chloroplasts in these crude preparations. The rods in the bundles were often much shorter than those protruding from the surface of the chloroplast fragments, which reached the well-known length of about 280 mμ. This was also the most common length of rods outside the chloroplasts in crude preparations.

Some small bodies were micrographed which appeared to be built up almost exclusively of bundles of short rods (Fig. 10). It was not possible to identify these bodies as chloroplast fragments. The rods in the bundles were about 80 mμ. As can be seen from Fig. 10, there is a parallel arrangement of the bundles, but the parallelism is not complete. These bodies show some similarity to X-bodies but the short length of the rods is remarkable.

In micrographs of preparations of sap from plants which had been infected for a long time, the rods were often found to be arranged in a semi-crystalline way. Many rods were then aggregated length-wise and these long aggregates were arranged around centres of crystallisation, thus giving fan-like figures in the micrographs. Such figures are probably due to tactoids in the sap.

In a mixture of a preparation of purified tobacco mosaic virus and a suspension of healthy chloroplasts, some rods were found to be concentrated in a similar way to chloroplast fragments which had obviously served as centres of crystallisation.

Potato X and some other filamentous viruses. In preparations containing potato X virus, the chloroplasts appeared to be badly damaged and were more fragmented than in the case of beet yellows and tobacco mosaic virus. The X virus threads were often connected with chloroplast fragments (Fig. 8). They were very often attached to small dense granules as was also observed in some preparations of tobacco mosaic virus.

Filaments attached to chloroplast fragments were also found in preparations of potato virus Y and from sap of leaves infected with *Pisum* virus and *Brassica mosaic* virus.

DISCUSSION

The above observations are submitted as evidence that at least some filamentous viruses are being formed in chloroplasts, and it would be of interest to try to verify the reported results by means of other methods.

The fact that virus rods and fragments of chloroplasts now and then occur associated is of course no proof of formation of virus in chloroplasts.

One has to take into consideration what kind of association there appears to be and what the rest of the preparation looks like. Thus, the examination here described of fragmented tissue infected with tobacco mosaic virus gave the expected results if one assumes that virus was propagated in some of the chloroplasts of the tissue and was released from those chloroplasts to the surrounding plasma. These free particles should then occur in the pictures without connection with the chloroplast fragments of the preparation or be associated with them only as a result of the random distribution of the two kinds of structures. (No adsorption was observed.) Chloroplasts producing virus may contain varying amounts of rods. Since the preparations for electron microscopy were made quickly and without any drastic treatment, there should be a good chance of getting well preserved fragments of such virus containing chloroplasts in the preparations which were studied in the electron microscope. The concentration of rods in such fragments and near to them should then be due to an accumulation of rods at and near to their sites of formation. However, the possibility can still not be excluded that pictures fitting into this scheme may be obtained as the result of a second hand attachment of the virus rods on to some fragments. Thus, the pictures published here do not give conclusive answer to the question as to the sites of formation of tobacco mosaic virus.

Tobacco mosaic virus has been reported to be produced also in tissues lacking chloroplasts (6, 16, and 14) so the green plastids of leaves cannot be the only site of formation of this virus.

SUMMARY

Examination with the electron microscope has revealed that the filamentous particles characteristic of leaves infected with beet yellows virus are frequently found in association with chloroplasts, and appear to have been extruded from them. Similar investigations have been carried out on tobacco mosaic virus. The assumption is made that at least some viruses are formed within chloroplasts.

My sincere thanks are due to Professor Arne Tiselius for his great interest and the excellent working facilities offered to me. I am also indebted to Dr. Daniel Lihnell, Stockholm, and Professor Karl Björling, Ultuna, Uppsala for valuable discussions and for provision of material.

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THE STRUCTURE OF CHLOROPLASTS

AN ELECTRON MICROSCOPICAL INVESTIGATION OF SECTIONS

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The chromoplasts of "higher" plants and green algae have long been considered to contain all the chlorophyll of these plants. Many investigations on their structure and function have been carried out after Sachs in 1862 asserted them to be the sites of the photosynthetic process. The literature on chloroplasts has been reviewed among others by Rabinowitch (27) and Strugger (33).

The inhomogenous, "granular" structure of the chloroplast has been much discussed, but was finally established by light microscopy (25, 31, 7, 2). Heitz described the "grana" as small disks having their flat surfaces parallel to the surface of the chloroplast (8). The very large chloroplast of the moss *Anthoceros*, photographed in ordinary light by Menke and Koydl (23) and in ultraviolet light by Menke (21), appeared to consist of stacks of laminae. These results were lately confirmed by Strugger (33) who, from light microscopical observations, described the chloroplast as consisting of a stroma without chlorophyll and chlorophyll-containing grana piled in stacks straight through the chloroplast. After swelling of the chloroplast in an alkaline solution, layers within it could be observed. Strugger's conception of the inner structure of the chloroplast has, however, been derived partly by indirect means and in some respects still remains to be verified experimentally. The existence of discrete grana of the kind described by Strugger within each stack, and the regularly arranged "Trägerlamellen" which should even have an inner lamellar structure, could not be verified in the studies described in this report. Important results have also been obtained from studies of the birefringence (30, 12, 18, 13, 36) and dichroism (24, 20) of chloroplasts. A negative monoaxial double refraction, the optical axis being normal to the surface of the chloroplast, was found in the plastids of the living cell. After imbibition with glycerol, this negative double refraction disappeared. It was therefore considered to be a morphic birefringence. The imbibed material on the other hand, showed a weak positive double refraction which was supposed to be due to oriented anisotropic molecules. These studies thus indicated a lamellar inner structure of the plastids, each

layer consisting of long molecules arranged parallel to each other and normal to the plane of the laminae. On this basis Hubert and Frey-Wyssling developed a hypothesis according to which the chloroplast is built up of alternating layers of proteins and lipids (9, 3).

Chemical analysis of isolated chloroplasts (19) have shown that these contain about 30 per cent lipoid material. The occurrence of peculiar protuberances, so-called myelin figures from the surfaces of plastids treated with solutions of detergents (35, 17) also indicated the presence of phospholipids, since lecithin was known to give such formations easily.

Production of starch inside the chloroplasts was observed as early as in 1862 by Sachs (28). Menke considered the laminae observed by him in the *Anthoceros*-chloroplast to be separated by accumulated assimilates (21).

The small size of chloroplasts called for the electron microscope as a means of revealing their inner structure, and papers on electron microscopical investigations of whole and fragmented chloroplasts have been published by several authors (10, 5, 1, 4). They all confirm the view of the chloroplasts as consisting of dense grana embedded in a stroma. When drops of suspensions of ruptured chloroplasts are dried and studied in the electron microscope, small rounded disks appear in large numbers. Most authors consider these disks as parts of grana which should thus have a lamellar inner structure. Algera *et al.*, however, think that the disks are the dried walls of small myelin vesicles (1). It has been observed by many authors that myelin protuberances may take in water through their thin walls and swell thus forming more or less spherical vesicles of varying size. Electron microscopical pictures of such vesicles are found in papers by Algera *et al.* (1), Granick and Porter (5), and Frey-Wyssling and Mühlethaler (4).

The opinion that the chloroplast is surrounded by a membrane in the living cell now appears to be generally accepted (11, 1, 5, 4, 33, 34). Frey-Wyssling and Mühlethaler have published an electron micrograph of what they consider to be an isolated membrane of this kind from a ruptured chloroplast (4). Thomas *et al.* (34) found the chloroplastic membrane to consist of proteins and lipids. A similar composition was described by him for a presumed membrane surrounding each granum.

The study presented here was induced by observations on leaves infected with beet yellows virus (15, 16). In the course of our investigations of virus and chloroplasts, some results concerning the structure of the plastids and the accumulation of assimilates were obtained which may be of general interest.

MATERIAL AND METHODS

Fragmented and whole chloroplasts. *Beta sacharifera* and *Aspidistra elatior* were cultured in glass houses. Leaves of these species were macerated by grinding in mortars. From the ground mass, sap was pressed through muslin. Serial 5-fold dilutions were made with distilled water. Drops of each dilution were placed on collodion-covered copper-grids for electron microscopy. After drying, the preparations were shadow-cast, usually with gold-manganin 1:1.

Sections. The leaves were placed in the fixing fluid and cut in pieces some millimeter thick. For a first fixation, the material was always kept in 0.4 per cent formaldehyde solution for half an hour. This solution had previously been neutralized to pH 6.9–7.1 with sodium hydroxide. After washing in running tap water during one hour, the specimen was dehydrated by means of ethanol or dioxane and embedded. Preparations treated in this way had fairly well preserved chloroplasts. The cytoplasm outside the plastids, however, was badly shrunk and could usually be found only round the plastids and near the cell walls.

After fixation in formalin followed by treatment with osmium tetroxide and chromium compounds, the cytoplasm of the cells was much better preserved. The sequence recommended by Grey, Klesch and Schüster (6) was applied and the following concentrations and fixation times were used: neutral formaldehyde, 0.4 per cent, 1/2 hr; running tap water, 1 hr; equal parts of osmium tetroxide, 0.3 per cent, and potassium dichromate, 0.3 per cent, 1 hr; chromium acid 0.3 per cent, 1 hr; running water, 2 hrs.

This procedure was followed by dehydrating and embedding. The sectioning was carried out according to Newman, Borysko and Swerdlow (26). The specimens were thus embedded in a methacrylic resin, a mixture of 1 part methyl- and 4 parts *n*-butyl methacrylate usually giving convenient hardness. Sections were cut with a glass-knife (14) in a standard Spencer microtome with a thermal expansion device cooled by carbon dioxide. The sections were treated with isoamyl alcohol and isoamyl acetate to dissolve the methacrylate. They were shadow-cast with palladium or gold-manganin 1:1.

RESULTS AND DISCUSSION

The chloroplast membrane. As can be seen from Fig. 1, sections in which the cytoplasm is preserved do not show any membrane round the chloroplast. The stroma of the chloroplast continues directly into the cytoplasm outside the plastid. If there has been a membrane, this must have been dissolved by the agents which fixed the cell components seen in the picture.

Fig. 3 pictures a whole chloroplast, not sectioned. Several preparations of whole chloroplasts have been micrographed by other authors (1, 4). This one was made to show the margin of the chloroplast. On the surface of the plastid several small "myelin figures" are to be seen but no sign

of a membrane. The objection might here be raised that the chloroplast may have been (partly) isolated from its membrane after the rupture of this latter. Such phenomena are easily observed when bacteria are studied under the electron microscope. Naked bacteria can be found together with their stripped off membranes (29). The nature of the bacterial membrane will not be discussed here. Concerning the chloroplasts, however, these form myelin figures very easily, not only of the type shown in Fig. 3 but also large myelin bladders. To the micrographs already published by other authors (5, 1, 4) Fig. 4 is presented here, since it may be valuable for the present discussion. A fragment of a chloroplast (white area in the midst of the picture) has given rise to three large myelin bladders and some small ones. (Disintegrated grana are also seen.) In Fig. 3 such large myelin bladders can also be seen on one side of the chloroplast. Frey-Wyssling and Mühlethaler (4) have published a picture of a partly empty "Hautbalg" of about the same diameter as that of a whole chloroplast. The present author has observed, by light microscopy, that chloroplasts when kept in distilled water or hypotonic sucrose solutions for several hours often give rise to water clear bladders of about the same size as chloroplasts.

The evidences accumulated up to now do not allow any conclusive answer to the question whether a chloroplast membrane exists or not. The following interpretation of the available data seems most plausible: The boundary between the chloroplast stroma and the surrounding cytoplasm may be characterized as a mere phase boundary, the chloroplasts being denser bodies within the cytoplasm without any membrane. When the cell is disintegrated and the composition of the phase outside the chloroplast is changed (a dilution with distilled water is sufficient, detergents are not needed), this change may cause a precipitation of the outer layer of the lipid-containing chloroplast stroma which thus forms a membrane as an artefact. This membrane is probably semi-permeable to the solution outside. As long as there are no holes in the membrane it will take in water, and the globe will swell by osmosis. Very often there will soon occur a rupture. Parts of the contents will then come out into contact with the outside medium giving rise to new bladders of the same type and of varying size. The bladder described by Frey-Wyssling as the "skin-membrane" of the chloroplast may have been formed by myelin formation at the surface of a chloroplast, whereafter the bladder has ruptured and been partly emptied.

The inner structure of the chloroplast. Fig. 1 shows the grana and stroma within the chloroplast. If the grana are built up of layers as is com-

monly assumed, a section in a plane normal to the one of Fig. 1 might be expected to reveal the lamellar structure of the grana. Figs. 2, 8, and 9 show such sections of two different species. They confirm the view of the grana as stacks of thin disks. The stacks are arranged with their long axes about parallel to each other and normal to the long axis of the chloroplast. This orientation is, however, not strict, there rather appears to be a closest packing in the chloroplast of stacks of different heights. In the rather few sections obtained with a lamellar structure clearly visible, 15–60 lamellae were found in each stack. The diameter of the disks was found to be 0.3–0.6 μ (i.e. the width of the broadest stacks seen in the sections, which are supposed to be sectioned through their long axes).

No doubt, most of the small disks in micrographs of fragmented chloroplasts are identical with the lamellae of the grana-stacks. In Figs. 5 and 6 such lamellae are shown, one series from *Beta* with about 20 disks, others from *Aspidistra*, of which one contains about 55 lamellae. The lamellae are very thin and special arrangements are probably needed for an accurate determination of their thickness. It appears however, to be far below the limit of resolution of the light microscope. Thus, the layers observed by light microscopy (33) cannot have been separate lamellae of the kind described here.

In Fig. 8 some lamellae can be seen continuing from one stack into another. Thus, all the stacks in the chloroplast do not seem to be completely isolated from each other. Several micrographs have been obtained from *Aspidistra* showing the same thing. Were it not for pictures like Fig. 1 one would assume, from the sections of *Aspidistra*, that these chloroplasts contain lamellae straight through the chloroplast in the direction of its long axis, only interrupted here and there by accumulations of starch. A more detailed knowledge of the structure of chloroplasts will probably be obtained after studies of cells of varying age and under varying physiological conditions.

The occurrence of accumulated assimilates in chloroplasts. So far there hardly exists any histochemistry on the level of resolution of the electron microscope. In spite of the monochromacy of the picture obtained by means of electrons, it seems theoretically possible to determine the chemical nature of structures seen e.g. in an electron micrograph of a section. Some structures may be selectively "stained" with ions of heavy metals and after such treatment show stronger scattering of electrons than the corresponding structures of non-treated controls. It may also be possible to remove some substances selectively from the sections by means of more or less specific solvents or

enzymes. A first attempt to use this technic has been made on fragmented chloroplasts by Menke (22) and Thomas *et al.* (34).

It is very probable from a physiological point of view, that the accumulated substance to be seen within the chloroplasts and often between two neighbouring plastids (Fig. 9) is starch. This substance in the sections shows no structure, except the granulation due to the metal layer from the shadow-casting (which granulation can be seen also on the membrane supporting the specimen). Sections of potato starch appeared very similar to the substance under discussion when studied in the electron microscope. In sections of chloroplasts, the substance appears in clumps between the stacks in varying amounts. In most chloroplasts several clumps of assimilates were found, in others only one appeared in the midst of the plastid. The assimilates may be accumulated in large amounts in both types and fill most of the space of the chloroplasts. The sections studied here gave the impression that the lamellae of a stack may be separated by layers of assimilate of varying thickness. It is true that variations in the direction of the planes of the lamellae with respect to the direction of the sectioning may falsely give this impression. However, some sections of chloroplasts which appeared to be completely filled with starch showed distinct, very thin lamellae within the masses of accumulated assimilates. More work has to be done before a complete description can be given of the occurrence and accumulation of assimilates, but the findings reported here do not, so far, support Strugger's opinion: "Photosynthese und Stärkekondensation verlaufen also in getrennten Räumen."

Fig. 7 is a section of the chloroplast of the moss *Anthoceros*. It shows a chloroplast with several clumps of assimilates arranged thus that they might give the impression of a lamellar structure. As mentioned above, Menke interpreted his photographs of the *Anthoceros*-chloroplast as showing lamellae separated by accumulated assimilates.

GENERAL CONCLUSIONS

Thus, the results obtained by electron microscopy of sectioned chloroplasts are consistent with earlier results obtained by electron microscopy of fragmented plastids, by indirect physical methods and by light microscopy of chloroplast structures which are so small that they are near and beneath the limit of resolution of the light microscope.

As pointed out by Thomas *et al.* (34) the structures of chloroplasts appear fundamentally similar to those of the retinal rods of the eye, studied

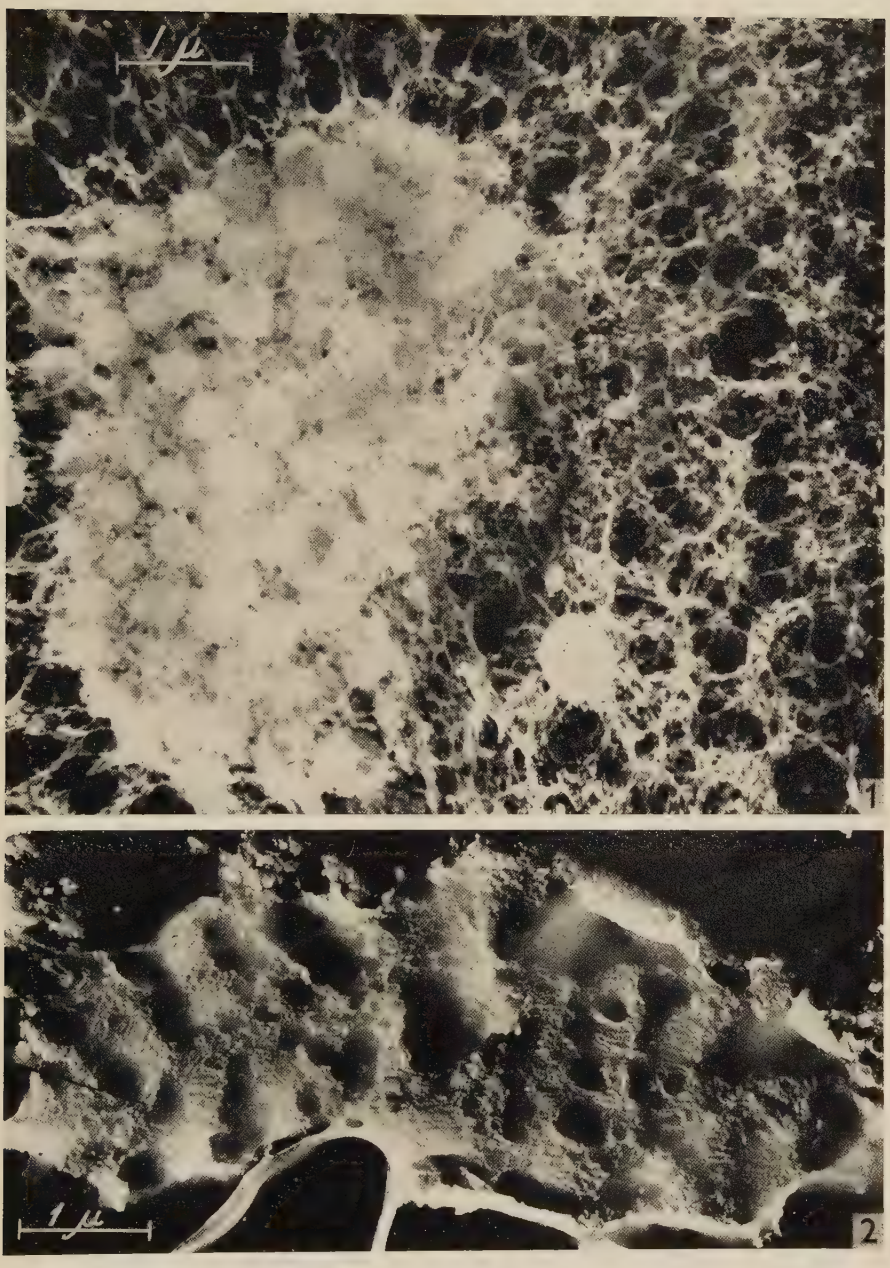
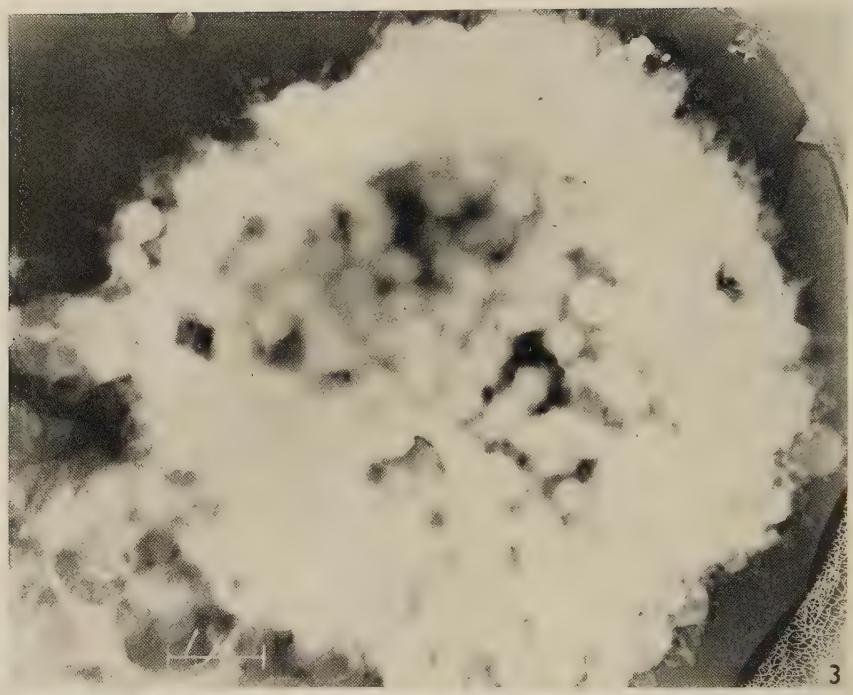


Fig. 1. Section of a chloroplast of *Beta sacharifera*, fixed in formaldehyde, osmiumtetroxide and chromium compounds.
Fig. 2. Section of a chloroplast of *Beta sacharifera*, fixed in formaldehyde solution.



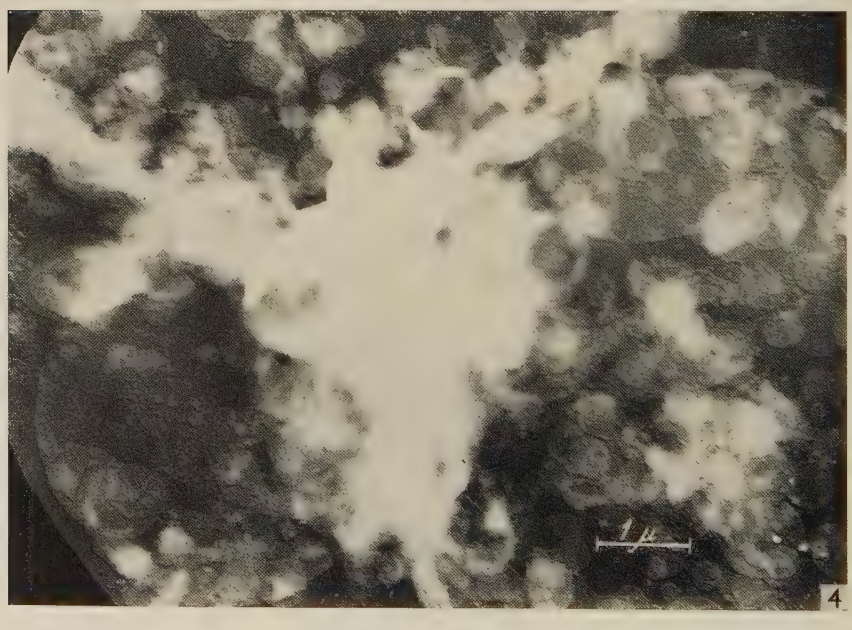


Fig. 3. Chloroplast of *Beta sacharifera* from a dried suspension.
Fig. 4. Fragment of a chloroplast of *Beta sacharifera*, with large myelin formations.

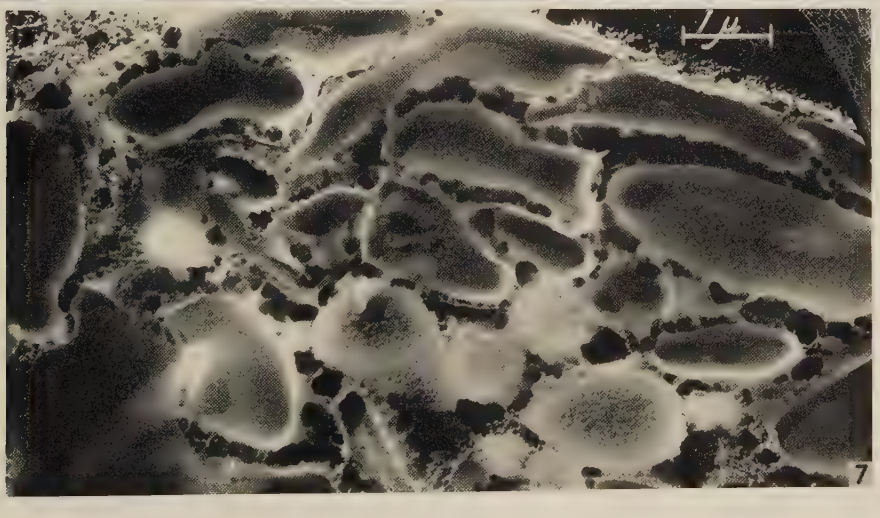


Fig. 5. Lamellæ of "granula" from chloroplasts of *Aspidistra elatior* and Fig. 6. *Beta saccharifera.*
Fig. 7. Section of a chloroplast of *Anthoceros sp.*

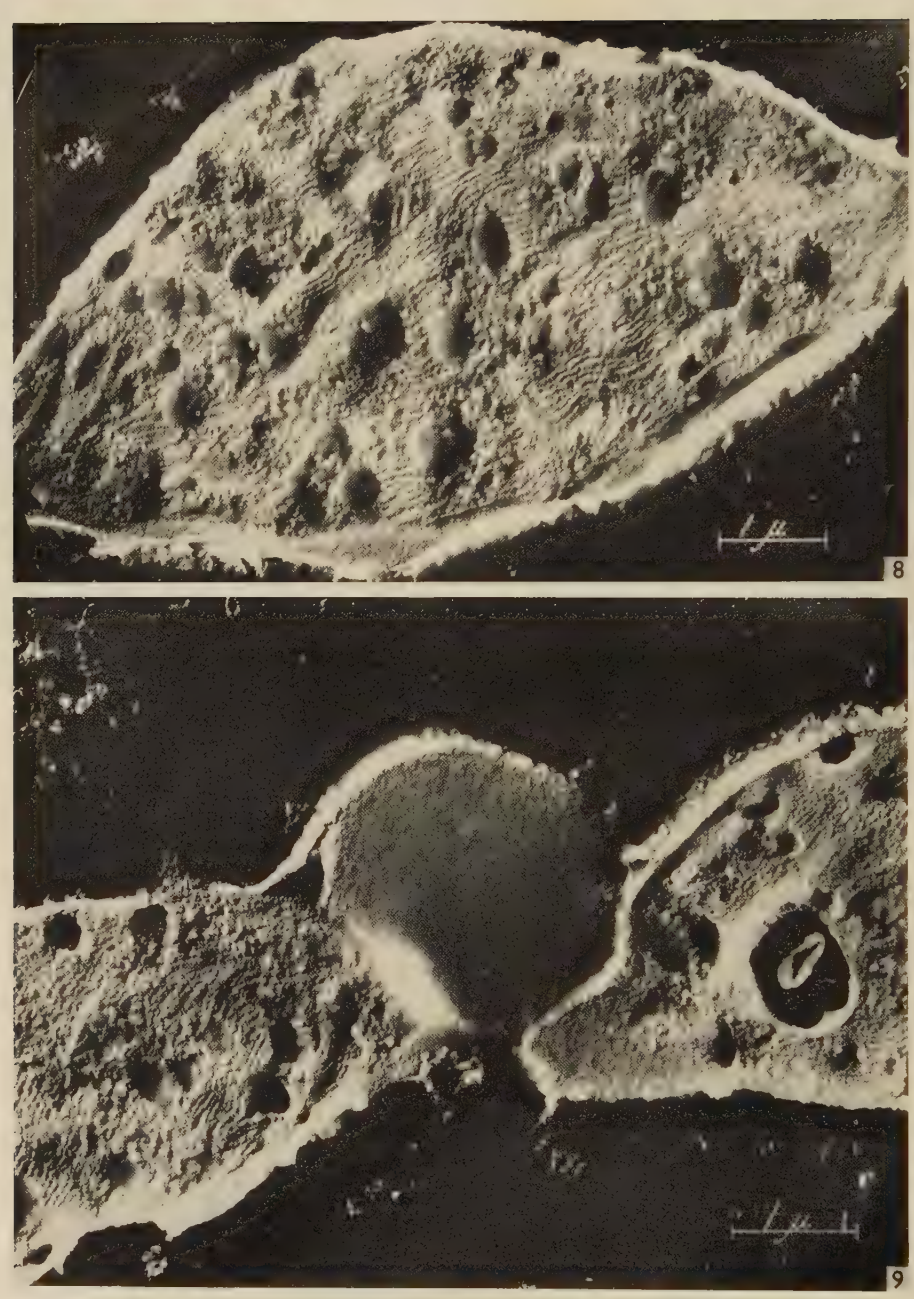


Fig. 8. Section of a chloroplast of *Aspidistra elatior*, fixed in formaldehyde solution.
Fig. 9. Accumulated assimilates between two chloroplasts of *Aspidistra elatior*. Fixed in formaldehyde solution.

partly by means of the same methods as for the chloroplasts. Electron microscopical investigations on the structure of the rods of guinea pig eye have been carried out by Sjöstrand (32).

SUMMARY

1. Electron microscopical pictures of sections of chloroplasts of *Beta sacharifera* and *Aspidistra elatior* are presented.

2. Available facts are interpreted to show no membrane of the chloroplast nor of the "grana" within the chloroplast.

3. The "grana" are shown to be stacks of lamellae, the long axes of the stacks arranged about normal to the long axis of the chloroplast.

4. The occurrence of accumulated assimilates within and outside the chloroplast is shown.

My thanks are due to Professor Arne Tiselius for his kind interest and for the facilities put to my disposal. The sectioning technic for electron microscopy was introduced here by Dr. E. Ribi and embedding and sectioning were performed by Mrs. K. Ribi. For this help, I also express my sincere thanks.

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THE SPECIFICITY OF THE GALLOCYANIN-CHROMALUM STAIN FOR NUCLEIC ACIDS AS STUDIED BY THE RIBONUCLEASE TECHNIQUE

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Basic dyestuffs, such as toluidine blue and methylene blue, are in the histological specimen bound to structures containing high molecular organic acids. With ribonuclease technique (2) it has been shown that ribose nucleic acids often form the basis for this staining. Structures that contain other high molecular acids, for example chondrin and heparinocyte granules, are also stained by basic dyes, although in a different manner, producing a metachromasy. As a rule, however, this metachromasy is easy to recognize and distinguish from the original colour of the dye solution.

The dyes mentioned stain in a *regressive* way, i.e. the sections are overstained and then differentiated to a suitable intensity. It is therefore difficult to get objective information on the structures. It is claimed that some of them can be used specifically for demonstrating nucleic acids (10, 15, 16). Quantitative photometric measurements have, however, not given satisfactory results (18) and all investigators do not agree that the dyes show a specificity (8, 20).

It seems to be of great value that a basic dye is available with *progressive* staining properties, viz. the gallocyanin-chromalum. With this dye the sections cannot be overstained and no differentiating is thus needed. By varying the pH of the staining solutions, the selectivity of the dye is changed (5, 6, 11, 12). According to Einarson (6), it is even specific for nucleic acids within a certain range of pH (0.8–1.75). This has, however, been denied by others (7).

For the reasons mentioned, it was thought desirable to study the hypothetical specificity of the gallocyanin-chromalum stain for nucleic acids by aid of the ribonuclease technique.

PRELIMINARY EXPERIMENTS

When incubating with various solutions, no substances must dissolve from the control sections incubated with the buffer solution alone. It was confirmed that 10

per cent formol was a suitable fixative in this respect (19), while Carnoy's fluid was most unsuitable. As the heparinocytes are better preserved in Carnoy-fixed than in formol-fixed specimens, the thyroid, which contains many heparinocytes, was fixed in Carnoy's fluid. It was not necessary to use the ordinary fixative for heparinocytes, 4 per cent basic lead acetate. The deparaffined sections mounted on glass slides were then immersed in 10 per cent formol for four hours and no substances were detected to dissolve in the control buffer.

MATERIAL AND METHODS

The study was performed on the following organs fixed in the following ways: pancreas, liver and spinal cord (all in 10 per cent formol), thyroid including heparinocytes (Carnoy's fluid), cartilage (10 per cent formol), vaginal epithelium of a 40-day-old rat, 48 hours previously injected subcutaneously with 100 I.U. α-oestradiol monobenzoate (Ovex B Leo) (10 per cent formol).

The pieces were embedded in paraffin and cut 7 μ thick. Sections from all organs were mounted on the same glass slide. They were deparaffined, brought through alcohols to water and then immersed for four hours in 10 per cent formol.

After thorough washing with distilled water they were incubated at 37° C for three hours in a solution containing 5 mg ribonuclease in 100 ml McIlvaine's standard buffer at pH 7.0 (3). The ribonuclease (crystallized, salt free, Worthington Biochemical Sales Co) was tested for its proteolytic activity by measuring its ability to liberate cyclic amino acids from hemoglobin (kindly performed by H. Emanuelsson). No proteolytic activity was observed.

Stains: The gallocyanin-chromalum stain was prepared by boiling 0.15 g gallocyanin (National Aniline Division) and 5 g chromium and potassium sulphate c.p. ($Cr_2K_2(SO_4)_4$, $24H_2O$) (5) in 100 ml distilled water in a reflux apparatus for 18 hours. Previous studies in this laboratory had shown that if gallocyanin and chromalum were boiled longer than usually (three minutes), the resulting solution gave a more intense staining without any detectable loss in progressivity or selectivity. After cooling the solution was filtered. Staining time 24 hours.

Toluidine blue (Hollborn) was used in 0.5 per cent solution. The sections were stained for 12 hours and then differentiated in 96 per cent alc. until the stain clouds were gone.

Methylene blue (Merck) was used in 0.5 per cent solution. The sections were stained for 12 hours, then washed in distilled water four times during two minutes and finally the stain fixed by immersing in 5 per cent ammonium molybdate c.p. for three hours.

Feulgen: The staining was performed according to Stowell (17).

All the solutions were prepared immediately before use. They were buffered to the desired pH:s with HCl c.p. and NaOH c.p. with the aid of a glass electrode (Mac Beth pH meter).

The final concentrations of toluidine blue and methylene blue were those mentioned above. The gallocyanin-chromalum solutions were diluted 10 per cent during the buffering.

Table I

	Gallocyanin-chromalum				Toluidine blue		Methylene blue	
	pH 1.0		pH 2.0–4.0		pH 3.0		pH 3.0	
	RN	C	RN	C	RN	C	RN	C
Ergastoplasmic zone of pancreas cells	–	+	+	+	–	+	–	+
Liver cell inclusions	–	+	+	+	–	+	–	+
Thyroid cell cytoplasm	–	+	+	+	–	+	–	+
Cytoplasm and keratohyaline granules of vaginal epithelium cells	–	+	+	+	–	+	–	+
Cytoplasm of intestinal epithelium cells	–	+	+	+	–	+	–	+
Nissl-substance of spinal cord cells	+	+	+	+	–	+	–	+
Chondrin and heparinocyte granules	+	+	+	+	+	+	+	+

RN = ribonuclease-treated for 3 hours.
C = control.
\+ = stained.
— = not stained.

RESULTS

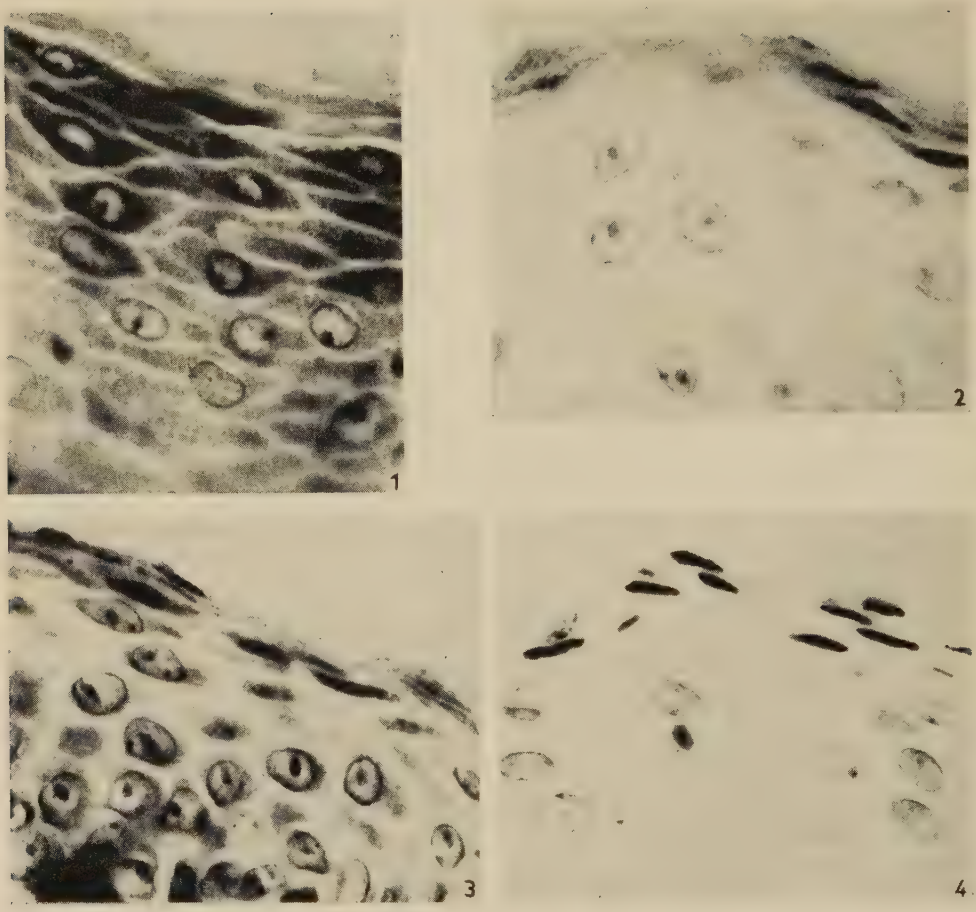
Metachromatical staining: At all pH:s and with all dyes used, chondrin and heparinocyte granules are well stained in a metachromatic colour. The staining is most intense at high pH:s. This is best seen in the peripheral zone of the cartilage where the appositional growth is taking place. Here cells may be colourless at pH 1.0 while they are stained at pH 4.0.

The metachromatic staining is not influenced by ribonuclease-treatment.

Non-metachromatical staining: Toluidine blue and methylene blue: At pH 1.0 the staining is very weak or absent. After ribonuclease treatment no staining of the cytoplasm. At pH 2.0 the staining is weak but distinct. After ribonuclease treatment no staining of the cytoplasm. At pH 3.0 the staining is very intense. After ribonuclease treatment no staining of the cytoplasm (see Table I). At pH 4.0 the staining is still more intense. After ribonuclease treatment there is staining of the cytoplasm.

Gallocyanin-chromalum: At pH 1.0 the staining is intense (Figs. 3 and 7). After ribonuclease treatment there is a weak but distinct staining of the Nissl-substance (Fig. 8). No other cytoplasmic structures are stained (Fig. 4). As it is claimed by Einarson (6) that the gallocyanin-chromalum is specific at this pH, some additional experiments were performed to ascertain this result.

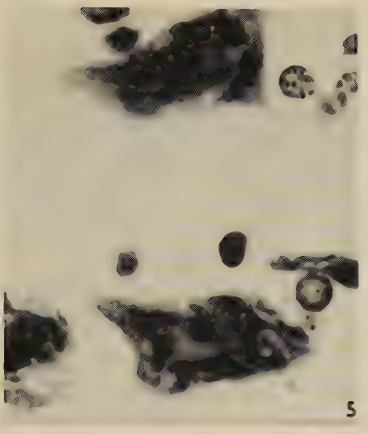
1) Rinsing with distilled water for half an hour did not diminish the stain-



Figs. 1–4. Vaginal epithelium. Gallocyanin-chromalum stain boiled for 18 hours. Fig. 1 stained at pH 4.0, control. Fig. 2. pH 4.0, ribonuclease-treated for 3 hours. Fig. 3. pH 1.0, control. Fig. 4. pH 1.0, ribonuclease-treated for 3 hours. Formol fixation. Thickness of section 7 μ. Photographed through a Farrand interference filter, peak wave length 574 mμ, half band width 16 mμ. Magnif. ×820. Immersion objective N.A. 1.0 apochromat.

ing. 2) Not even with 25 mg ribonuclease per 100 ml buffer and an incubation time of 48 hours was it possible to remove the staining though it became slightly weaker. 3) The dye solution was prepared by boiling as Einarson (5, 6) recommends for three minutes. Then the staining was much weaker

Figs. 5–9. Spinal cord. Figs. 5–8. gallocyanin-chromalum boiled for 18 hours. Fig. 9. gallocyanin-chromalum boiled for 3 minutes. Fig. 5. stained at pH 4.0, control. Fig. 6. pH 4.0, ribonuclease-treated for 3 hours. Fig. 7. pH 1.0, control. Fig. 8. pH 1.0, ribonuclease-treated for 8 hours. Fig. 9. pH 1.0, ribonuclease-treated for 8 hours. For technical details see Figs. 1–4.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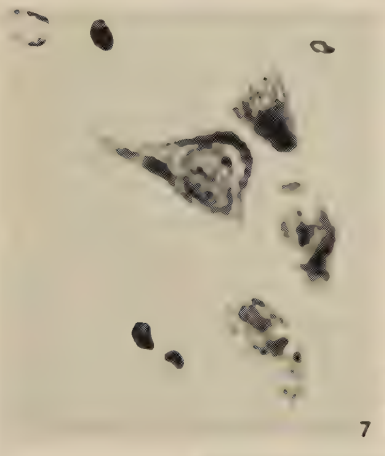
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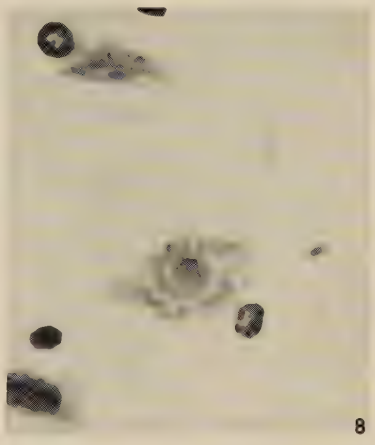
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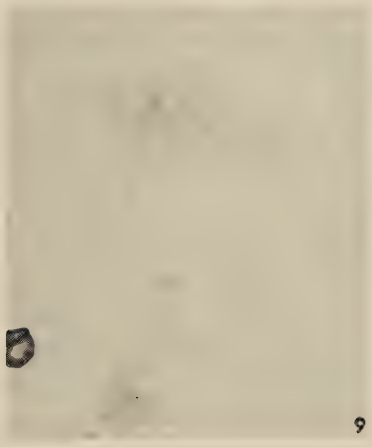
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(Fig. 9) but not completely lost even when incubating for 48 hours with 25 mg ribonuclease per 100 ml buffer. All other organs were also stained with this dye solution. The short-boiled solution gave a weaker staining than did the long-boiled. This difference was especially marked in the metachromatic staining of chondrin at pH 1.0. The ribonuclease experiments gave the same results with both dye solutions.

At pH 2.0–4.0: With rising pH values the staining becomes more intense. More and more cytoplasmic structures are stained after ribonuclease treatment (Figs. 1, 2, 5 and 6).

DISCUSSION

As mentioned in the introduction the metachromatic colour of basic dyes is easily distinguished from the "ordinary" colour. Therefore no attention is given to metachromasy in the following discussion, though it cannot be excluded that difficulties may arise if *one* structure is stained in *both* ways.

Nucleic acids are present in the nuclei as ribose nucleic acids (RNA) and as desoxyribose nucleic acids (DNA). The DNA is specifically demonstrated with the Feulgen technique (17). Therefore a) staining after ribonuclease treatment with a dye specific for nucleic acids, and b) staining according to Feulgen, should give identical pictures. The identity is, however, not easy to prove, and the results presented here did not necessitate such evidence.

Nucleic acids are present in the cytoplasm only as RNA. Staining after ribonuclease treatment should therefore give no staining of the cytoplasm with a dye specific for nucleic acids. This is easily confirmed by observations.

The gallocyanin-chromalum stain did, however, give a cytoplasmic staining after ribonuclease treatment at pH 1.0 when this dye is said to be specific for nucleic acids (see Table I). This result may be interpreted in different ways.

1. Remains of nucleic acids are present in the nerve cell. These are stained only by gallocyanin-chromalum and not by toluidine blue or methylene blue. a) Danielli (4) has pointed out the possibility that a core of nucleic acids may be surrounded by a layer, perhaps only a monolayer, of protein. The enzyme can thus not obtain access to the substrate and the nucleic acids remain undigested. b) Tsuboi and Stowell (22) studied the effect of the same ribonuclease as used in this investigation on RNA from mouse liver cytoplasm and found that only part of the RNA was digested. The same holds true for yeast nucleic acids (13). It is possible that the denaturation, taking place when isolating the substances or when fixing histological

material may change the digestibility of the nucleic acids so that these lose their specific properties (1).

2. Toluidine blue and methylene blue are specific but so is not gallocyanin-chromalum. Thanks to its progressivity, however, the use of gallocyanin-chromalum together with ribonuclease offers excellent opportunities for demonstrating RNA. It is therefore worth while attempting to make this staining more specific. The staining substance of the boiled gallocyanin-chromalum solution should be isolated. The "non-specific" staining might then disappear. This staining may also have an adsorptive character. One might then be able to differentiate it or wash it away, although the advantage of the progressivity would be lost. This may no doubt be a method for a specific staining of nucleic acids (9, 14, 21).

It is not known which of these explanations is correct. Further investigations are planned in order to solve the problem.

SUMMARY

It could not be shown by aid of the ribonuclease technique that the gallocyanin-chromalum stain is specific for nucleic acids, the metachromatic staining not being considered. Toluidine blue and methylene blue seemed to stain specifically at pH 3.0. The technique is discussed.

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THE DIFFERENTIATION OF CELL WALL, CYTOPLASMIC MEMBRANE AND CYTOPLASM OF GRAM POSITIVE BACTERIA BY SELECTIVE STAINING

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Many species of bacteria are plasmolysed by strong solutions of salts (7, 14, 18), and the process may be reversed when the organisms are returned to a normal medium. In plant and animal cells this property of semipermeability is attributed to a thin layer at the surface of the protoplast, which has been termed variously the "plasma membrane" or the "cytoplasmic membrane." This membrane contains a relatively high concentration of lipoids (3, 13, 24). Because bacteria can be plasmolysed a similar surface structure may be postulated and evidence for the presence of a cytoplasmic membrane has been assembled by Knaysi (20). Methods of selective staining of such a surface layer being unsatisfactory or uncertain, we were led to a trial of the Victoria blues 4 R and B.

The Victoria blues (V. blues) are water soluble basic dyes of a deep shade belonging to the phenol-methane series. They differ from most of the basic dyes in general use because they are almost completely extracted from aqueous solutions when shaken up with lecithin or fatty acids in ether as shown by Schumacher (36). In experiments with fixed bacteria we have observed that dilute aqueous solutions of the V. blues pass readily through the cell wall, staining it lightly, and are *arrested at the surface of the protoplast* endowing it with intensely stained and sharply drawn contours. This behaviour differs markedly from that of the customary basic dyes (such as crystal violet, methylene blue, thionine and fuchsin), which immediately stain the substance of the protoplast and have no selective affinity for its surface layer or for the cell wall. We feel that a description of these and related observations may be of help to other students of the structure of bacteria.

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MATERIALS AND METHODS

The organisms used were *B. megaterium*, *B. cereus* and a large, filamentous *Bacillus* "M" which resembles *B. cereus* var. *mycoides* but failed to give the egg yolk reaction (22). Gram-negative bacteria particularly *Escherichia coli*, have been examined for comparison. A yeast was isolated from bakers yeast (Fleischman's) and maintained on yeast extract-glucose-agar. The other organisms were grown on heart infusion agar.

Fixation. Thin films of young bacterial growth on heart infusion agar plates were fixed and more or less quantitatively transferred to No. 1 cover glasses by the method of fixation through the agar with Bouin's fluid (15, 17, 25, 31, 32, 40). Agar blocks were cut from the plate and placed, cells down, on cover slips. These were then immersed in Bouin's fixative for an hour (longer fixation does no harm), when the blocks were flicked off the cover slips. The fixed impression films were washed in water before staining. Samples of the heavy growth of older cultures were thinly spread on the surface of water agar and fixed in the same way.

The yeasts required different treatment. They were grown for 18 hours on the surface of a yeast extract-glucose-agar plate. A small block was then cut out from the agar and exposed, cells up, to osmium tetroxide vapor for 3 minutes in a closed vessel. The agar cut-out was next inverted on a cover slip on which a loopful of egg albumin in water (5–10 ml egg white shaken up with 100 ml of distilled water) had previously been allowed to dry. With the yeasts now facing the dried albumin, the cover slip carrying the agar block was placed in a dish with 35 per cent alcohol —1 per cent acetic acid. A mixture which, in the course of 20 to 30 minutes, transferred most of the yeasts from the agar to the cover slip.

Fixation through the agar has the important advantage over the drying and heating of bacteria used in diagnostic routine procedures that it leaves them in a condition which permits sharper differentiation of the parts of the cell by selective staining than is possible after drying. Comparison of living with fixed or supravitally stained cells has convinced us that fixation through the agar with Bouin's mixture preserves the shape, size and internal configuration of bacteria very well.

The separation of cell and protoplast was obtained by three methods, each with their special usefulness as will be noted later: 1) plasmolysis, 2) fixation of dried cells and 3) the fixation of cells immediately or soon after a brief exposure to ether vapour.

1) Plasmolysis was most easily obtained by placing cells in a drop of 5 per cent potassium nitrate spreading it and allowing the film to dry. To stain these preparations with aqueous dyes without reversal of the plasmolysis required fixation in 70 per cent alcohol. This is essentially the method of Fischer (7). For observation of plasmolysis of the living cell a modification of the method of Knaysi (18), diffusing 25 per cent sodium chloride through a thin agar block, seemed the most useful.

(2) A useful amount of partial shrinkage of the protoplast away from the cell wall was induced in impression preparations of young cultures of *B. megaterium*, *B. cereus* and *Bacillus* "M," which were allowed to dry in air for 5 minutes and were then fixed for 5 minutes or longer in a saturated solution of picric acid in

water or in Bouin's solution. The extent of the shrinkage so produced is much greater than that observed after drying in air alone. The best results, in very many experiments, were consistently obtained with 2–3 hours old plate cultures of *B. megaterium* and, especially, *Bacillus* "M" grown on heart infusion agar at 37° C. Failure to produce shrinkage in cells from much older cultures (8 hours) was equally consistent.

3) Exposure of young plate cultures of *B. cereus* or *Bacillus* "M" to vapors of ether, acetone or chloroform for 5 to 10 minutes, is soon followed by the lysis of many or most of the cells. However, fixation immediately after seven to ten minutes exposure to ether vapor and *before* lysis had set in, produced a result that was useful for our purpose. In many bacilli fixation caused very clear separation of cell wall and cytoplasmic surface. In some instances, e.g. in *bacillus* "M," this was mainly achieved through a relaxation of the cell wall, in others there was also some shrinkage of the protoplast. The effect, which is not seen when normal cells are fixed through the agar, is most pronounced around the margin of the preparation. In other experiments young plate cultures were first exposed to ether vapor for some 10 minutes, then removed from the ether and left standing, open, in air. After direct microscopy of the growth on the agar had indicated a useful amount of lysis, sample cut-outs were fixed through the agar with Bouin's solution while the remainder of the growth was washed off with 5 per cent formalin, centrifuged twice and washed with distilled water. From this suspension of fixed cells specimens were prepared for electron microscopy. The usefulness of autolysis for the purpose in hand has already been recognized by Knaysi (20) in his studies on the cytoplasmic membrane of bacteria and is reaffirmed by the electron micrographs of lysing bacilli illustrating the present paper.

Yeasts in a more or less advanced stage of cytolysis were obtained by suspending young growth in saline to which a few crystals of thymol had been added and keeping it for 18 hours in the incubator at 37°. The lysing yeasts were centrifuged, spread thinly on the surface of a well-dried agar plate, fixed with osmium vapor and subsequently treated like the normal culture.

Staining. Selective staining for the analysis of cell structure is not obtained with the customary strong solutions of dyes because most bacteria become deeply and evenly stained throughout in a very short time. Under these conditions the V. blues differed in no respect from other dyes; the difference from other dyes was only apparent when dilute stains were applied for a short time. Accordingly, fixed films were rinsed and then stained for 20 to 40 seconds only in 0.01 per cent thionine or crystal violet or fuchsin in water and compared with films stained for the same length of time in 0.05 per cent V. blue B or 4 R in water. Stained films were rinsed, mounted in water, sealed with wax and examined with an oil immersion lens through the cover glass.

In water mounts V. blue soon diffused (5–10 min.) from the cell surface into the cytoplasm until useful differentiation of surface and interior was lost. Treatment of fixed cells with a saturated solution of mercuric chloride in water for five minutes before staining considerably retarded the inward diffusion of V. blue (while only slightly delaying the entry of ordinary dyes into the cytoplasm) and was found very useful for the photography of stained preparations. Following mercuric chloride, ordinary dyes, e.g. thionine, may also selectively stain the cell surface. But

the effect is neither as general nor as sharp and brilliant as that obtained with the V. blues.

Water mounts were a distinct advantage in this work because the cell wall, unless it is very intensely stained, becomes completely invisible if mounted in oil or Canada balsam.

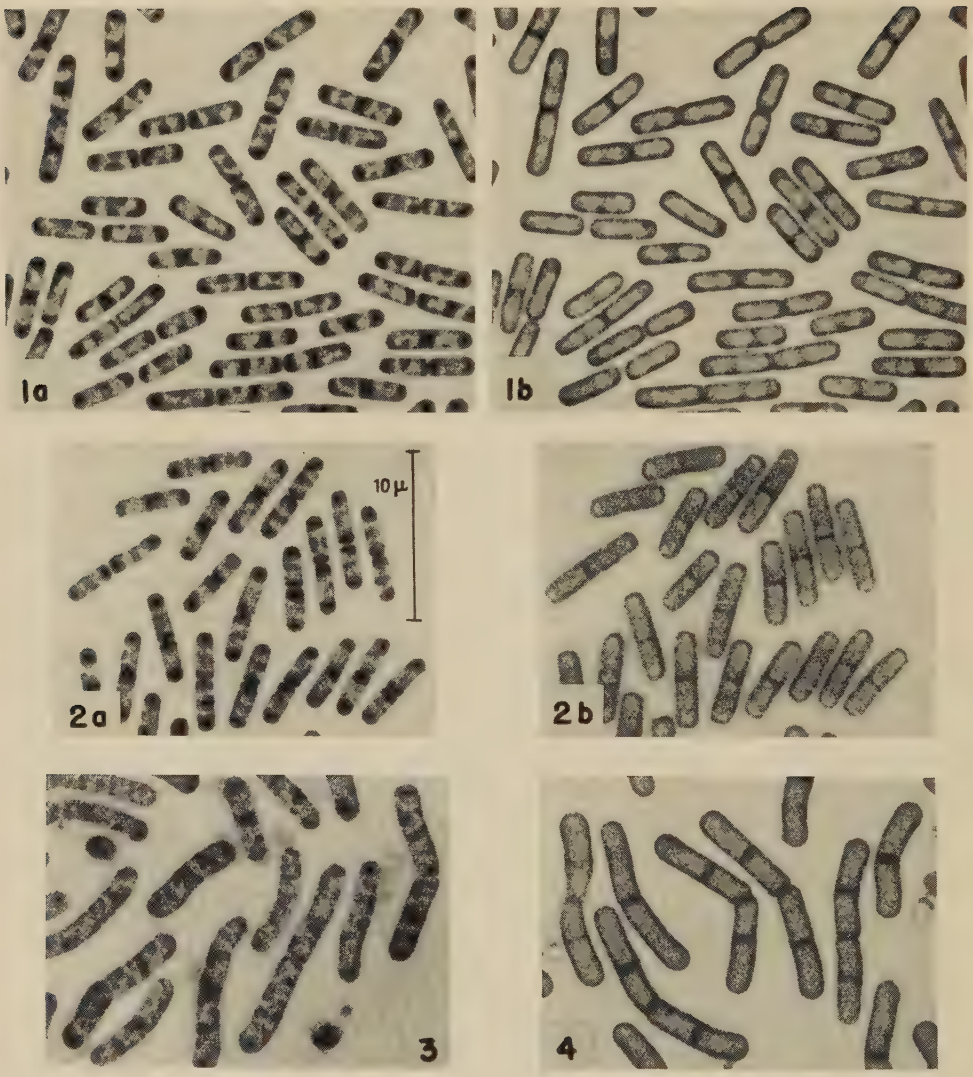
Photomicrography. A Bausch and Lomb tungsten ribbon research lamp was adjusted to provide Koehler-type illumination (Method II of Shillaber (37)). For direct observation an achromatic substage condenser, n.a. 1.4 (Zeiss), oiled to the slide, and an ×90 apochromatic oil immersion, n.a. 1.3 (Zeiss) was used in conjunction with an ×15 compensating eye piece. A green (5390 A) interference filter (Baird Associates, Cambridge, Mass.) was used for taking most of the photographs. The latter were made on Kodak Panatomic-X sheet film at an initial magnification of 1800–2000 × and enlarged 1 1/2-2 times in making positive prints.

OBSERVATIONS

Properties of the Victoria blues. Schumacher (36) studied the behaviour of aqueous solutions of V. blue B and of thirty other basic and acid dyes when shaken up with lecithin or fatty acids in ether. He noted that V. blue was almost entirely extracted from water by the lipoid solutions, while the other dyes proved either insoluble in the lipoid phase or distributed themselves in varying proportions between the two phases. In comparative tests with the two V. blues (B and 4 R), crystal violet, fuchsin, methylene blue and thionine, we have convinced ourselves of the correctness of Schumacher's observations on V. blue B, but did not find V. blue 4 R behaving very differently from ordinary dyes under the conditions of the lecithin test. Both dyes were, however, equally readily and completely extracted from water by shaking with chloroform[1], in which crystal violet is only moderately, and thionine not at all soluble. Another property that we have observed is a marked surface activity. Aqueous solutions formed a stable froth when shaken. Further, a small amount added to a "cleaned" tray of water formed a surface film that became visible when its area was restricted by a moving barrier. Quantitative measurements of surface activity have not been made.

Staining of bacteria with Victoria blue. When fixed bacteria were stained with V. blue they differed in two respects from bacteria that have been stained with ordinary dyes. In the latter the cytoplasm was deeply stained. Within the cytoplasm were regularly spaced areas filled with unstained or

[1] V. blue B turns red when entering chloroform from water but is blue when added directly to the solvent.



Figs. 1 and 2. Comparison of the effects of direct staining with thionine (a) and with Victoria blue 4 R (b) on identical groups of *B. cereus* fixed with Bouin's fluid. (a) shows unstained nuclear structures in the deeply stained cytoplasm. (b) shows the same group stained with V. blue after removal of the thionine with acid-alcohol. The dark contours and septa of the bacilli are formed by cytoplasmic membrane and cell wall in close contact.

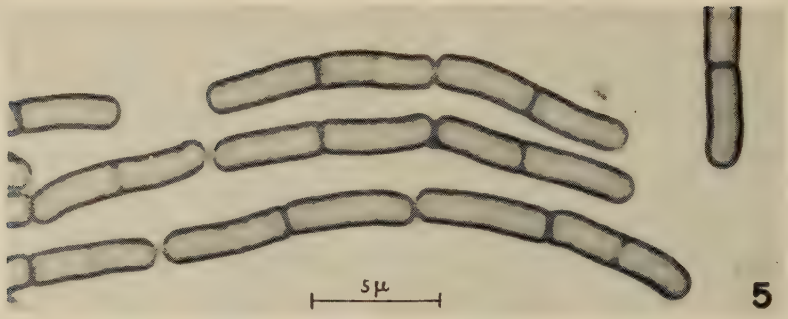
Fig. 3. *B. megaterium* fixed with Bouin's fluid and stained for 20 seconds with 0.01 per cent basic fuchsin.

Fig. 4. Bacilli from the same culture fixed in the same way as in Fig. 3, but stained with Victoria blue 4 R.

only faintly stained material. These areas, from other tests, are known to be the sites of the nuclear structures (17, 31, 32). V. blue stained neither the nuclei nor the cytoplasm, but deeply coloured a very superficial layer of the cell which ordinary dyes stained only after prolonged application. This difference between the results of staining with ordinary dyes and with V. blue is demonstrated in Figures 1–4. Less easily described is a subtle change in the overall optical properties of bacteria stained with V. blue which showed itself as a curiously glossy appearance of the cell (Fig. 5). It was evident that V. blue not only stained the cell surface but at the same time markedly affected its refractility.

As noted in Methods, selective staining with V. blue required a low concentration of the dye. After a few minutes the stain started to diffuse from the protoplasmic surface into the cytoplasm (cf. lower half of Fig. 1 b) and, although mercuric chloride pretreatment slowed the diffusion, examination immediately after staining was necessary. These characteristics of V. blue were consistently observed with the *Bacillus* species studied. Representative species of *Lactobacillus*, *Proprionibacterium*, *Corynebacterium* and a number of strains of *Listeria monocytogenes* behaved in exactly similar fashion. However, organisms such as *E. coli* and others of the enteric group did not show any selective staining of the superficial layers and the action of the V. blues did not differ from thionine and other stains.

The sharply stained contours with which V. blue initially endows bacteria were formed by two separate layers normally in close contact: namely, *cytoplasmic surface* ("cytoplasmic membrane") and *cell wall*. The double nature of the cell contour was apparent wherever the protoplast had retracted from the cell wall. A few cells in this condition may be observed in normal preparations (40), but the separateness of cell wall and cytoplasmic surface was more clearly revealed when extensive retraction of the protoplast was induced by plasmolysis (Figs. 12 and 13), drying or ether treatment before fixation (Figs. 8 and 11). After these treatments, V. blue stained the cytoplasmic boundary as deeply and sharply as the contours of normal bacilli (Figs. 6 and 8). The cell wall of dried-fixed cells was only faintly stained and of a delicate thinness just visible in water mounts, largely due to its refractility. Its thinness in this kind of preparation was of the same order as the unstained cell wall of bacilli plasmolysed in the living condition. Ether pre-treatment gave similar results as far as the protoplast was concerned but there was a difference regarding the *cell wall*. The latter was often unusually thick, in preparations stained with V. blue (Fig. 11), and no longer as thin as that of living, plasmolysed bacilli. Thus, although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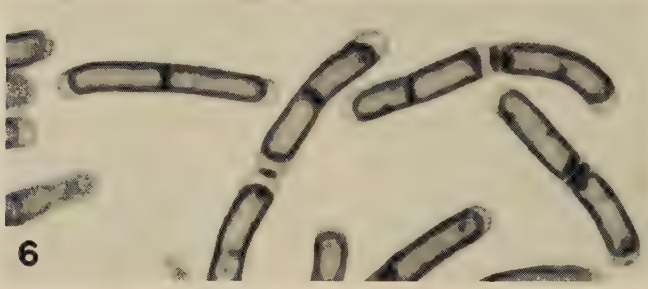
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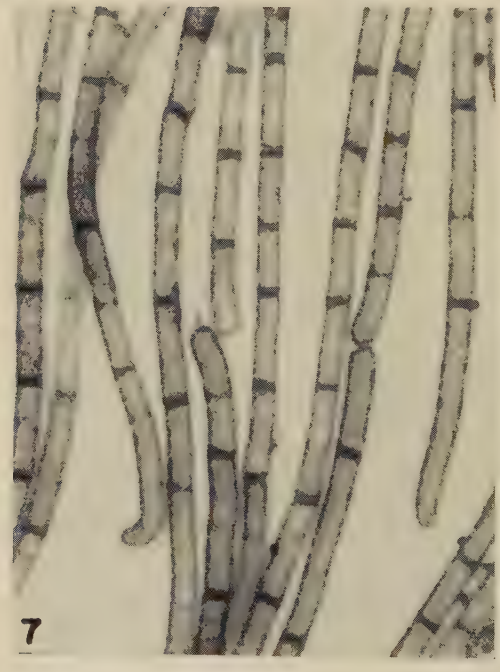
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the ether treatment was useful in showing the double nature of the V. blue stained contours of normal cells, it caused some alteration in the structure of the cell wall, not seen after the other treatments. We infer from these observations that in fixed and stained normal bacilli, most of the V. blue at the cell surface is in the cytoplasmic membrane.

Brief exposure to ether vapor was of further value because the lysis which follows was accompanied by the disappearance of the superficial layer at which V. blue is normally arrested. Lysing cells, stained by V. blue, did not look different from lysing cells stained with thionine, crystal violet or other ordinary basic dyes. This showed that V. blue could stain the interior of the protoplast like other basic dyes provided it was allowed to penetrate. The same change has been seen during lysozyme-lysis of *B. megaterium* (40).

The concept of a special structure, a cytoplasmic membrane, which normally impedes the diffusion of V. blue to the cytoplasm received support from the demonstration, in electron micrographs (which we owe to the kindness of Dr. V. E. Cosslett and Mr. G. E. Crowe of the Cavendish Laboratory, University of Cambridge) of remnants of a thin membrane at the surface of protoplasts of *B. cereus* after brief exposure to ether vapor (Figs. 9 and 10). This membrane was quite distinct from the cell wall and resembled the thin, partly destroyed layer that Knaysi (20) demonstrated in "ghost" forms (spontaneous lysis) of *B. megaterium*: a layer which he too regarded as a remnant of the cytoplasmic membrane.

V. blue stained the cytoplasmic membrane in the same way at all stages of the growth cycle, whereas the underlying cytoplasm lost much of its basophilia and changed in other ways as the culture passed out of the logarithmic phase of growth. This provided further evidence of the special character of this surface layer.

Staining of cytoplasmic surface and cell wall with Sudan black B. Knaysi

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Fig. 5. *B. megaterium* treated with mercuric chloride after fixation and stained with V. blue B. Staining is sharply restricted to the cell surface.
Fig. 6. *B. megaterium* dried in air for 5 minutes, then fixed with Bouin's solution; treated with mercuric chloride and stained with V. blue B. In every bacillus there has been retraction of the protoplast from the cell wall, particularly from the free rounded ends of the cells and away from transverse septa. The cell wall is more faintly stained than the cytoplasmic membrane.
Fig. 7. *Bacillus "M"* treated as in Fig. 5.
Fig. 8. *Bacillus "M"* stained with Victoria blue B after six minutes exposure to ether vapor followed by Bouin-fixation through the agar. Relaxation of the cell wall has caused separation from the cytoplasm. Protoplasts slightly shrunken along their long axis. Note transverse septa. The cytoplasmic membrane is more deeply stained than the cell wall.

(20) has published a photograph in which the surface of *B. megaterium* appears deeply and selectively stained with Sudan black B and regards this result as evidence of the lipoid content of the plasma membrane. Cell wall and protoplast had evidently not been separated and the photograph suggests that the cell wall had been stained as well as the plasma membrane. We have stained *B. megaterium* with Sudan black B on many occasions and like others have been impressed by the sharp contrast between the deep staining of even the smallest of fat droplets and the complete lack of colour in the cytoplasm, but we have never obtained a picture comparable to that published by Knaysi (20). With 0.5 per cent Sudan black B in ethylene glycol (11, 38) we have repeatedly obtained faint, but distinct staining of cell wall and cytoplasmic surface in *B. megaterium* in which the two layers had been separated by Method 1. A result not unlike a faint V. blue preparation. We hesitate, nevertheless, to accept this, *a priori*, very plausible, result as a direct demonstration of lipoid material in cell wall and cytoplasmic surface because it took a very long time, more than 24 hours, before the effect of the stain was clearly noticeable; also under these conditions, we were equally successful in imparting a distinct gray or brownish alcohol-fast stain to fine tissue paper, so-called "lens paper," thought to be free from demonstrable amounts of lipoid material.

Observations on yeast cells. The cytoplasmic membrane of yeast cells can be stained with V. blue B, but the dye is not arrested at this layer without the prior use of a mordant. We have found 5 minutes treatment with a saturated solution of mercuric chloride quite effective after osmium fixation, much less so after fixation with Bouin's solution. Selective staining of the cytoplasmic membrane of yeast cells is also achieved by Eisenberg's tannic acid method (6) and Heidenhain's iron alum-hematoxylin.

Fig. 15 shows that in normal yeast cells V. blue was arrested at the surface of the protoplast. The entry of V. blue into the cytoplasm of the lysing cells was not impeded despite pre-treatment with mercuric chloride (Figs. 16 and 17), presumably owing to the destruction or severe alteration of the surface layer. There was shrinkage of the protoplasts and the cell wall was more deeply stained than in normal yeasts. A similar increase in the affinity of cell wall of lysing cells for V. blue has already been pointed out above for *B. cereus*, fixed after exposure to ether vapor.

The changes in the structure of yeast cells incubated in saline in the presence of thymol are very similar to those which attended exposure of (Schizo-) yeasts to a cetyl-zephirol in the experiments of Meisel and Umanskaya (23).

DISCUSSION

The results of direct staining with V. blue resemble, and should be compared with, those obtained when bacilli are first treated with a solution of tannic acid and then stained with crystal violet or some other basic dye. This is the method that Eisenberg (6) introduced for the staining of the "ectoplasm" of bacteria and that was used extensively by Gutstein (9, 10). The method has been revived and, with trifling modifications, used in a number of cytological studies (1, 2, 12, 16, 17, 32). It is based on the fact that the cytoplasm can no longer be stained with basic dyes after tannic acid treatment, while the affinity of the superficial layers for the same dyes is not impaired and may be increased. Differentiation of the superficial layers from the underlying cytoplasm is thus made possible. Eisenberg (6) also achieved selective staining of the cytoplasm when pretreatment was with mercuric chloride instead of tannic acid. We have used pretreatment with mercuric chloride to delay the inward diffusion of V. blue from the cytoplasmic surface and noted, as mentioned above, that the affinity of the substance of the protoplast for ordinary dyes is, indeed, decreased and that of the cytoplasmic membrane slightly increased by this treatment. Staining longer than usual (e.g. 45 seconds instead of 15 seconds with 0.01 per cent thionine in water) restores the normal picture, however, and useful selective staining of the cytoplasmic surface with ordinary dyes was not obtained by this method.

The term "ectoplasm" has rarely been used in the same sense by successive investigators and the resulting confusion has impeded the formulation of clear concepts of bacterial cell structure. A comparative study of the various meanings attached to the word "ectoplasm" will be presented elsewhere. Here it will suffice to point out that the terms "ectoplasm" as used by Eisenberg (5, 6) and "cell wall" as used by Robinow (32) refer not to a single anatomical entity, but to the optical effect produced by the superimposed images of the deeply stained cytoplasmic surface and the lightly stained cell wall in close contact (cf. criticism of tannic acid—crystal violet as a "cell wall method" (21, 33, 40). Eisenberg (5) noted that occasionally the ectoplasm is found to have split into two layers, "Membran" and "Rindenschicht" (i.e. cortex) corresponding to "cell wall" and "cytoplasmic membrane" of our terminology, but was not aware that a similar and reversible separation of the two layers occurs in plasmolysis. Both Eisenberg and Gutstein regarded bacteria as naked protoplasts held in shape by a two-ply ectoplasm and not as cells whose tough and chemically

inert walls enclose a detachable protoplast bounded by a semipermeable, chemically active plasma membrane. This latter concept, long ago put forward by A. Fischer (7) on the basis of his observations on plasmolysis in large spirilla and in small gram-negative organisms, has since been supplemented by plasmolysing experiments (14, 18) on *Bacillus* cells, and is now accepted as valid for many species of bacteria.

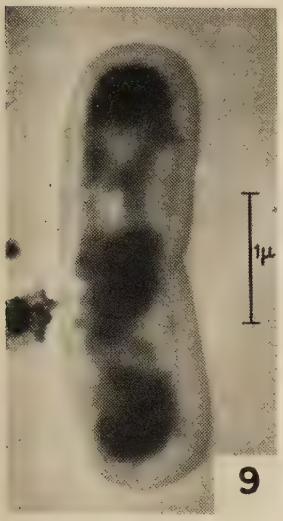
Some features of the changes in cell structure initiated by the methods that we have used for the separation of protoplast and cell wall are worth discussing in detail. The glossy sheen of V. blue stained cells is more noticeable in air-dried, post-fixed, and in ether-treated (but wet-fixed) cells than it is in normal bacilli. This similarity extends also to the cytoplasm which stains with thionine in a dull, diffuse manner after either treatment and lacks crispness of stain, transparency and the sharp delineation of the nuclear sites which are characteristic of the stained cytoplasm of fixed normal bacilli. Another effect of drying in air as well as exposure (of living bacilli) to ether or chloroform, manifests itself in a more striking manner: in both instances most of the bacilli disintegrate when incubation on a nutrient medium is resumed after the treatment. Lysis, especially after chloroform treatment, is rapid in *B. cereus*; slow, but inexorable, in *B. megaterium*. This observation reveals that the unusual brightness of the layer with special affinity for V. blue, the dull staining of the cytoplasm and the separation of the protoplast from the cell wall in a fixative which normally preserves bacilli well are all subtle indications of a severe disturbance in the cell's fine-structure brought about as effectively by a seemingly harmless drying in air as by exposure to ether vapor (not followed by drying).

Figs. 9 and 10. Electron micrographs of *B. cereus* fixed with 5 per cent formalin at an early stage of ether-induced cytolysis. In several places a thin membrane, distinct from the cell wall is seen stretched out over part of the surface of the disintegrating protoplasts. Comparison with normal bacilli in the left lower corner of Fig. 10 reveals that lysis is accompanied by considerable relaxation of the cell wall cylinder.

Fig. 11. *B. cereus* fixed through the agar with Bouin solution immediately following 10 minutes exposure to ether vapor. Treated with mercuric chloride solution, stained with Victoria blue B. In many cells the protoplast has retracted from the cell wall. This (useful) artefact of fixation is not seen when normal cells are fixed in the same way. Inset: the central bacillus of this figure more highly enlarged.

Figs. 12 and 13. *B. megaterium* fixed in the plasmolysed state according to Fischer (7) and stained with V. blue B.

Fig. 14. A chain of *Bacillus "M"* dried in air, then fixed with Bouin, treated with mercuric chloride and overstained with V. blue B to render the cell wall visible. Shrinkage of the protoplast has exposed the outer cell wall and transverse septa, recalling the description of shrunken cells of *B. anthracis* given by A. Fischer (7) and Eisenberg (5).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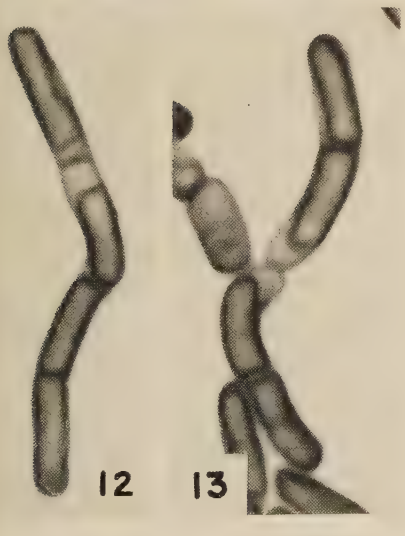
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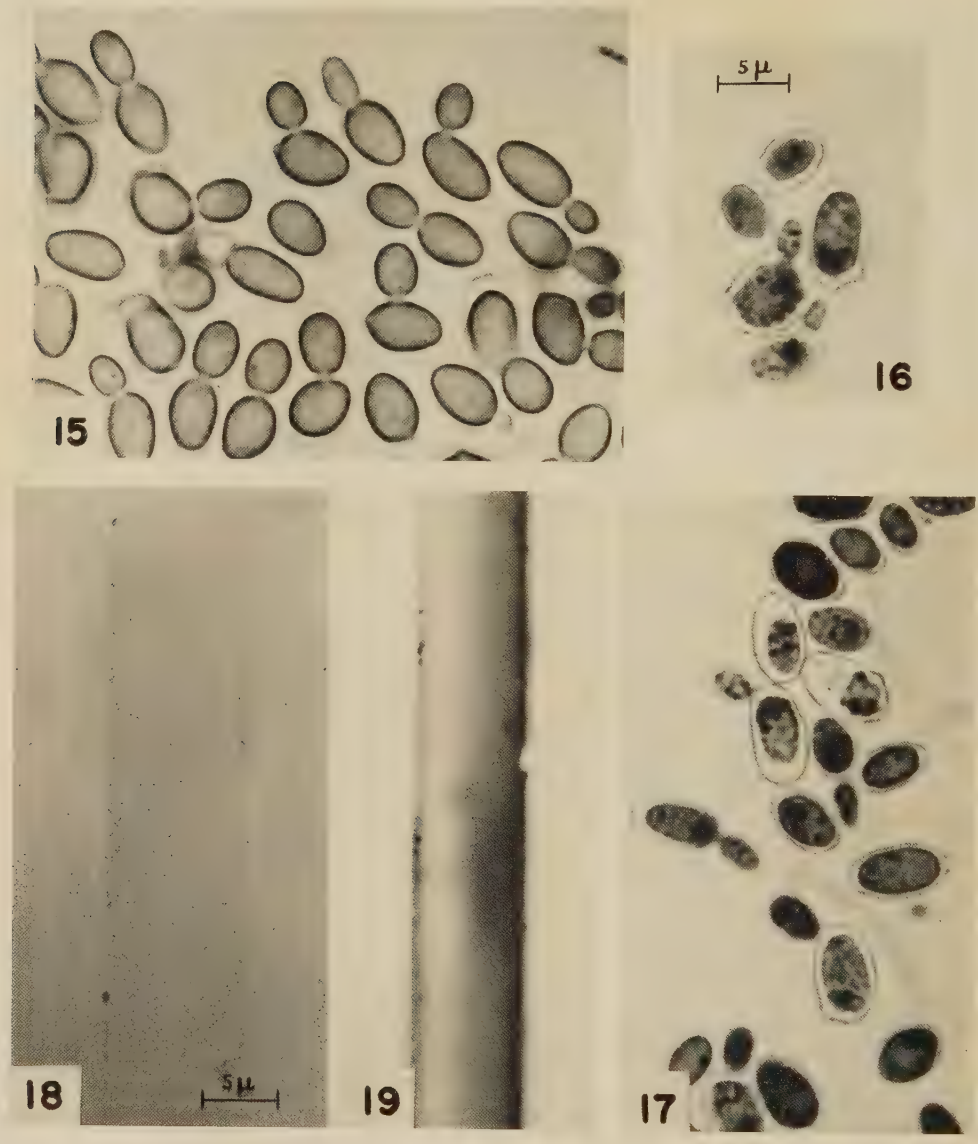

Fig. 15. *S. cerevisiae* fixed with osmium tetroxide vapour followed by alcohol-acetic acid fixation through the agar. Treated with mercuric chloride and stained with V. blue B. The cell wall is only very lightly stained and the dye has been arrested at the cytoplasmic membrane.
Figs. 16 and 17. *S. cerevisiae* showing autolysis due to incubation in saline in the presence of thymol. Fixation and staining as in Fig. 15. The surface layer at which V. blue is normally arrested seems largely destroyed, and in most cells the dye has penetrated to the interior.
Figs. 18 and 19. Fibres of "Pyrex" glass wool, photographed in Canada balsam. The fibre in Fig. 18 is untreated and could only be rendered visible by keeping the aperture of the substage condenser low. The fibre of Fig. 19 had been shadowed with gold before it was mounted. This increased its visibility so that it could be photographed with full Koehler illumination.

The failure of V. blue to reveal a surface layer in Gram-negative organisms is shared by the Eisenberg technique and still awaits explanation.

Wet fixation, followed by staining with one ordinary basic dye, preferably thionine, and with V. blue provides much information about the structure of *Bacillus* cells that is not readily obtained by other methods. Cytoplasm, plasma membrane and cell wall can be separately demonstrated and the nuclear structures, although they remain unstained in the procedures we have used, are clearly outlined in the cytoplasm. Lastly, a matter which it would be out of place to discuss here, the manner of division of these cells is very clearly revealed.

Methods such as we have used, which cause a separation of cytoplasmic membrane and cell wall, should give as much information about the one as about the other. In this paper we are chiefly concerned with the cytoplasmic membrane, but a few general remarks about methods for the staining of the cell wall might nevertheless be useful here. We believe that the only method which selectively demonstrates the cell wall without optical interference from the underlying structures (i.e. which effectively distinguishes the cell wall from the underlying structures without the need of special treatments) is that of Margaret Dyar (4). Eisenberg's technique stains both the cytoplasmic membrane and the cell wall, the former more strongly than the latter. In Knaysi's (19) tannic acid-alum-carbol fuchsin method the cell wall takes on a bluish sheen but is not always easily distinguished from the deep red protoplast with which it is normally in close contact. Before the cell wall can be clearly distinguished as a separate layer by any but the Dyar method, it is necessary to separate it by some means from contact with the underlying cytoplasmic membrane. When this is done and the stained preparations are examined in water, the cell wall appears very thin and has the perfectly smooth, sharp contours which it has during life in plasmolysed bacilli and with which we are also familiar from electron micrographs. In comparison with this type of preparation the aspect of the cell wall produced by the Dyar method appears rather unnatural, because the red precipitates that form in and on the cell wall considerably thicken it and may even throw it into conspicuous folds. It follows that the Dyar method is most useful when dried films have to be examined in oil but that other procedures are preferable when water mounts can be made.

The thickness of the layer in which V. blue is arrested is certainly much less than it appears to be, probably of the same order of magnitude as the thickness of the cell wall. Reflection from and absorption of light in the

curved sides of the only superficially stained cylindrical bacilli combine to give the illusion of deeply stained contours which make V. blue preparations so pleasing to look at. A similar striking difference in the apparent thickness of a thin stained membrane when extended in a plane at right angles to the observer and when viewed tangentially is apparent in photographs of empty, lightly stained cell wall cylinders of *B. cereus* (30). Here cross sections and tangential views of the cell wall simulated considerable thickness while the flatly extended torn edges of the cell wall sleeve were hardly perceptible.

Mitchell (24) has calculated a thickness of about 10 mμ for the osmotic barrier at the "so-called cytoplasmic surface" of bacteria. Experiments with ordinary and gold-coated microscopical glass fibres, carried out with the assistance of Dr. J. Hillier at the R. C. A. Laboratories at Princeton, N.J., suggest that a thickness of this order of magnitude would also account for the optical properties of the layer which V. blue reveals at the surface of the protoplast of bacterial cells. In these experiments "Pyrex" glass wool fibres, ca. 10 μ in diameter, were mounted in Canada balsam. Owing to the similarity between the refractive index of the glass and the resin, the fibres were invisible unless the aperture of the condenser was kept low. On another sample of fibres a layer of gold, not thicker than 20–30 mμ was deposited in a vacuum and these too were mounted in balsam and examined, this time with the full aperture of the substage condenser. The contours of the coated fibres were clearly visible, as if darkly stained, while the transmission of light through the fibres was not greatly diminished. The gold shadowed cylindrical fibre in Fig. 19 has a diameter of approximately 7.5 micron. The deposition of gold amounting to 1/250 of this thickness has produced an effect which simulates the action of V. blue on bacteria. On the assumption that similar proportions hold for a V. blue-stained *B. megaterium*, 1.5 μ thick, we arrived at a value of 6 mμ for the thickness of the cytoplasmic membrane. An estimate which is in good agreement with the 10 mμ advanced by Mitchell (24). The thickness of the cytoplasmic membrane shown in the electron micrographs of lysing bacilli also is compatible with this estimate.

Under the difficult optical conditions obtaining at the surface of normal, *living*, unstained bacilli mounted in a watery medium identification of cell wall and cytoplasmic membrane is not possible. The *cell wall*, but not the cytoplasmic membrane, becomes visible without recourse to staining as a separate layer when the protoplast of living cells retracts from the wall during plasmolysis, but retraction of the protoplast from the cell wall

does not suffice for the selective demonstration of the *cytoplasmic membrane*. For instance: in Salton's and Horne's (34, 35) electron micrographs of bacteria completely extruded from their cell walls by a heat treatment, the cytoplasmic membrane cannot be distinguished from the protoplast. The *cytoplasmic membrane* can only be distinguished from the underlying cytoplasm and from the cell wall when selective staining (e.g. with V. blue) is combined with retraction (natural or induced) of the protoplast from the cell wall, or when the cytoplasm has been destroyed as in ghost forms (20) or in bacilli disintegrating after exposure to ether vapor.

A cytoplasmic membrane about 300 mμ thick with "jagged or wavy inner surface" and comprising nearly half of the total thickness of the protoplast which Knaysi (20) has described and photographed in living *B. cereus* does not agree very well with the results that we have obtained with V. blue, is not in keeping with general concepts of the nature of plasma membranes, and does not fit the evidence from electron micrographs. Knaysi's careful account becomes understandable, however, when it is taken to refer, not to the cytoplasmic membrane as understood in the present paper, but to the optical effect produced by the contrast between the rather dense cytoplasm and the more transparent nuclear structures. A glance at Figure 1 a where this difference is enhanced by staining, will bear out this interpretation.

Why V. blue should have a selective affinity for the cell surface is not entirely clear from the structural formula of the dye. The affinity of V. blue for lecithin in ether and for chloroform, which was the starting point of our investigation, cannot be invoked as an explanation of the useful properties of the dye since it is also shown; if to a lesser degree, by other dyes, such as crystal violet and fuchsin, which do not primarily stain cell wall and cytoplasmic membrane (36). Moreover, V. blue 4 R is by no means as "lipophilic" as V. blue B, yet it seems nearly as useful for our purpose. It is more likely that an explanation of the manner in which V. blue is arrested at the cell surface will be found through the pursuit of the observation that the dye is markedly surface active. Chemical studies on more or less pure cell wall preparations have been reported by Weidel (39) and by Salton and Horne (35). The preparation of pure cytoplasmic membranes is probably impossible. However, the low affinity of the cell wall for dyes permits us to regard a superficial staining of bacteria, e.g. that obtained with the Eisenberg technique or with V. blue, as reflecting chiefly some property of the cytoplasmic membrane. Based on this reasoning and on the comparison of the behaviour of wool and of bacteria in var-

ious staining procedures, Fischer and Larose (8) have advanced novel ideas about the chemistry of the cytoplasmic membrane which are likely to stimulate further experimental work in this field.

SUMMARY

Low concentrations of V. blue 4 R and B stained the superficial layers of fixed *Bacillus* and yeast cells; under similar conditions ordinary basic dyes stained the cytoplasm only. Gram negative organisms did not respond in this fashion.

The avidity of the cytoplasmic membrane for V. blue was demonstrated when protoplast and cell wall were separated before staining. Separation was achieved by plasmolysis, drying or treatment with ether and subsequent fixation. The *cell wall* is only lightly and transiently stained by V. blue; most of the dye was arrested at or in the cytoplasmic membrane.

Electron micrographs of partially cytolysed *B. cereus* showed a thin membrane, distinct from the cell wall, stretched out over part of the cytoplasm. This was regarded as a remnant of the cytoplasmic membrane.

Victoria blue was also useful for the selective staining of the cytoplasmic membrane of a baking yeast. But there was not the primary affinity of the cell surface for V. blue which is seen in bacteria and treatment of fixed yeasts with mercuric chloride was necessary to demonstrate the cytoplasmic membrane. In yeasts in which autolysis had been initiated by incubation in saline in the presence of thymol a cytoplasmic membrane could no longer be demonstrated and in such cells V. blue readily stained the whole cytoplasm.

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CYTOCHEMICAL STUDIES OF THE ACTION OF TRYPSIN

III. THE COURSE OF DEFORMATION OF SALIVARY-GLAND CHROMOSOMES[1]

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The effects produced by treating fixed and unfixed salivary-gland chromosomes of *Drosophila* with solutions of trypsin are described in this paper. It supplements the first report of the series (11), in which the course of tryptic hydrolysis was outlined, and interprets the observations in accordance with the evidence presented in the second report (16) that trypsin causes cellular deformation by altering the gel-like properties of the constituent nucleoproteins. This paper also includes an appraisal of some of the earlier interpretations of salivary-gland chromosome organization in the light of the experimental evidence now available.

Disintegration of salivary-gland chromosomes of *Drosophila* may be effected by solutions of either crude or crystalline trypsin (3, 4, 19, 20). Such structural deformation has generally been attributed to the degradation of chromosomal proteins into diffusible fragments by the action of the enzyme, and the consequent liberation of the associated nucleic acid (3, 8, 19, 20, 25).

In appraising this interpretation, the question arises whether disruption by trypsin of specific peptide or amide linkages would in itself necessarily lead to disintegration of the chromosome, which has been shown in studies on fixed cells to be a highly complex fabric of proteins and nucleic acids (12, 14). It might be assumed that proteins would be so firmly combined with nucleic acids in the cellular nucleoproteins that digestion with trypsin would not lead to complete disintegration and loss of the proteins. Desoxyribonucleohistone is not completely degraded by trypsin, for amino acids have been found in dialyzed tryptic digests of this nucleoprotein (5). Also relevant to an interpretation of the action of trypsin on fixed cells are some observations made in this laboratory on isolated chromosomes and artificial "sections" of desoxyribonucleohistone (unpublished data of

[1] This study was aided by a grant (PHS RG-149) from the National Institutes of Health, U.S. Public Health Service.

Dr. Margaret McDonald). Hydrolysis of such material by buffered solutions of trypsin after "fixation" in Carnoy's mixture of acetic acid and alcohol (the reagent most commonly used in preserving cells that are to be subjected to enzymatic treatment) effected only partial digestion of the protein component, as determined by chemical analysis or stainability with acidic dyes.

In the light of these experimental findings, a critical examination of the course of chromosome destruction by solutions of trypsin seemed desirable. Evidence accumulated in recent years has shown that electrolytes may in themselves alter the appearance and staining reactions of cellular materials (10, 15, 26). Since trypsin has generally been used cytochemically in a solution of electrolytes, it becomes necessary to evaluate their action as well as that of the enzyme molecules in effecting alterations of cell structure. This has been done in the studies of salivary-gland chromosomes of *Drosophila melanogaster* here reported, in which the action of the enzyme in various solvents at different pH's was determined by treating intact glands and chromosome smears.

MATERIALS AND METHODS

The salivary-gland chromosomes used in this study were dissected from third-instar larvae of *D. melanogaster* in physiological salt solution. The detached glands were either kept in this medium until the trypsin or control solution was added (in order to determine the action of the enzyme on chromosomes of unfixed cells), or transferred to 45 per cent acetic acid for fixation (preparatory to determining the action of the enzyme on the chromosomes of fixed cells). Some of the fixed preparations were retained as intact glands, and others were converted into chromosome smears. In the latter event, the glands were crushed between slide and cover while immersed in acetic acid, and this temporary mount was transferred to 95 per cent alcohol for removal of the cover. The detached covers with the adherent cellular materials were passed through graded alcohols to water, and were then placed in the enzyme or control solution.

The fixed and unfixed preparations of whole glands were treated either in small watch crystals or in shallow chambers prepared as described below. The first of these methods permitted determination of the general nature of the induced changes by examination from time to time with low magnification; the second method permitted continuous examination of the cells at high magnification with either the phase or standard microscope. The smears could also be observed during treatment by using the slips to which the chromosomes were attached as covers of the shallow chambers. When the general nature of the reaction pattern had been established by such direct observation, the results were confirmed by the customary procedure of treating the smears in staining dishes.

In preparing the treatment chamber two grooves or troughs were cut with a

grinding wheel into a glass slide so that they would extend within and beyond the lateral edges of the cover slip when it was applied. The cover slip was supported by narrow strips of "Parafilm" (or paper impregnated with petroleum jelly) placed parallel and close to the upper and lower edges of the slide. The chamber formed in this manner was of sufficient depth to permit rapid exchange of fluids without displacement of the salivary glands from the field of microscopic observation, especially if the preparation included the bulky head and thoracic segments of the larva, the paired glands remaining attached to the pharynx. The grooves in the slide facilitated the exchange of fluids in the space between slide and cover as one reagent was replaced by another. Liquid was in some cases added to the grooves or removed therefrom with a medicine dropper, but a more rapid exchange was effected by applying thin strips of absorbent paper at one edge while introducing liquid at the opposite edge. The grooves also increased the amount of liquid that could be held close to the cover without overflowing its edges. This reduced the chance of evaporation of fluid, whose loss from the treatment chamber would have permitted the cover to press down and deform the underlying soft tissues of the gland.

The trypsin used was extracted from beef pancreas and crystallized in this laboratory by the method of McDonald and Kunitz (17). It was dialyzed against 0.0025 N HCl or water to remove the inorganic salts employed in its preparation, and was subsequently lyophilized from the frozen state. This salt-free, crystalline preparation was assayed chemically to determine its proteolytic activity and the possible presence of any contaminating nucleases (which represented less than 1 part in 100 000). The trypsin was used cytochemically in a concentration of 0.1 mg per ml (after a preliminary survey of the action of concentrations ranging from 0.01 to 10.0 mg per ml) dissolved in 0.05 M acetate or phosphate buffers of various pH's, in 0.05 M K_2HPO_4, or in H_2O (the pH was then adjusted by addition of a few drops of 0.01 N NaOH). This series included conditions of treatment that had been reported by previous workers to cause complete dissolution of salivary-gland chromosomes in fixed and unfixed cells.

In a few experiments, treatment of the chromosomes with either desoxyribonuclease or ribonuclease preceded treatment with trypsin. The nucleases were prepared in this laboratory; details concerning their purity have been published elsewhere (13). Desoxyribonuclease was used in a concentration of 0.02 mg per ml dissolved in 0.003 M $MgSO_4$ at pH 6; ribonuclease in a concentration of 0.1 mg per ml dissolved in H_2O and adjusted to pH 6 with 0.01 N NaOH.

All treatments were carried out at 37° C. The preparation under observation was kept on the stage of a microscope that was enclosed, except for the protruding binocular body tube, in a transparent plastic box equipped with sliding doors to permit access to controls for adjusting objectives, condenser, and stage. Circulating air, blowing over a thermostatically controlled heating unit, served to maintain constant temperature throughout the box, so that the slides (or watch crystals containing whole glands) could be temporarily transferred from the microscope stage to an enclosed storage rack without undergoing marked temperature deviations while other preparations were being examined.

Preparations of whole glands and chromosome smears were treated in the trypsin or control solutions for periods of time ranging from 1 minute to 16 hours. When

a 16-hour exposure to the action of the enzyme failed to produce marked alteration in the appearance of the chromosomes, digestion of other specimens was continued for 24 or 48 hours. To insure continuing potency of the enzyme over the longer periods of time, the solutions were renewed at intervals of 2 to 3 hours throughout the course of an experiment, since parallel chemical assay had shown that at 37° C a 0.01 per cent solution of trypsin at pH 7.5 or 8 may lose up to 90 per cent of its initial activity within 2 hours.

The preparations that had been treated as intact glands were stained upon removal from the trypsin or control solution in orcein dissolved in 45 per cent acetic acid (aceto-orcein), and were then made into permanent squash preparations. The salivary-gland-chromosome smears were stained either in aceto-orcein or by the Feulgen method. In those experiments in which desoxyribonucleic acid (DNA) was removed from the chromosomes with desoxyribonuclease before hydrolysis in trypsin, the specimens were stained in an acidic dye, such as fast green or light green, so that the chromosomes could be identified by the coloration of the residual protein material.

OBSERVATIONS

Action of Trypsin on Unfixed Cells

Chromosomes of glands transferred directly from physiological saline to buffered solutions of trypsin showed no evidence of enzymatic degradation during the first few hours of treatment. In many cases the cells separated from each other during treatment as a consequence of removal of intercellular cementing materials, but squash preparations of these cells in aceto-orcein revealed intact chromosomes with characteristic patterns of banding. Such maintenance of structural integrity is presumably due to inability of the enzyme molecules to enter the living cell, as was noted several years ago by Northrop (22, 23). Degenerative changes eventually occurred in the chromosomes of unfixed cells immersed in solutions of trypsin, and probably resulted from alterations in membrane permeability that permitted the enzyme to enter the nucleus. Bacterial contamination appeared to hasten the onset of such degenerative changes, since very few preparations in which bacteria were discernible showed unaltered chromosomes. Uncontaminated preparations, on the other hand, occasionally revealed chromosomes that were normal in appearance even after 24 hours of treatment. The possibility that degenerative changes in these unfixed cells were initiated by intracellular enzymes seems remote, because comparable alterations in structure did not occur within similar periods of time when the living cells were immersed in paraffin oil. Mazia (18) noted that chromosomes of salivary glands which remained under toluol for 7 days showed no signs of autolytic digestion at the end of that period.

Action of the enzyme in buffered solutions. The effect of trypsin in phosphate buffer at pH 7.5 on unfixed glands is shown in Figs. 1–6. The first 5 photographs show part of a gland at the same magnification during the course of treatment. As the enzyme or control solution replaced the physiological salt solution, swelling occurred (Fig. 2). No marked changes occurred thereafter in the appearance of glands immersed in buffer alone, but glands immersed in the trypsin solution soon showed indications of separation of cells (Fig. 3). At the end of an hour the glands appeared as loose aggregates of cells (Fig. 4), many of which subsequently drifted off into the surrounding medium as spherical units (Fig. 5). After 1 or 2 hours of treatment, some of the cells showed protruding vesicles filled with a transparent fluid, indicating extrusion of cytoplasmic materials (Figs. 4, 5). The treated cells were soft and easily deformed by pressure, but when fixed by drawing aceto-orcein under the cover into the treatment chamber, and then squashed, they revealed intact chromosomes that could readily be identified by their patterns of banding (Fig. 6).

Similar effects were observed when unfixed glands were treated with trypsin dissolved in phosphate buffer at pH 6, 7, or 8, or in K_2HPO_4 at pH 9. The action of the latter solution was tested because Mazia and Jaeger (19) had reported that a 0.1 per cent solution of crystalline trypsin in 0.05 M K_2HPO_4 effected complete dissolution of salivary-gland chromosomes of *D. melanogaster* within 3 to 4 hours. No such dissolution occurred when our preparation of crystalline trypsin was used for this period of time under the conditions specified. In a few cases it was possible to maintain the cultures as long as 8 hours—the limiting factor being the loss of cells by separation from the gland—and clearly defined chromosomes were then apparent in cells stained with aceto-orcein.

In another publication, Mazia (18) reported that digestion of salivary glands in trypsin at about pH 5 rapidly dissolved the chromosomes. Efforts were made to parallel the conditions of these earlier experiments by using the enzyme in acetate buffer at pH 5. Such treatment effected slight separation of the salivary-gland cells, but did not cause their disaggregation. Chromosomes with characteristic patterns of banding were observed in squash preparations of glands that had been treated as long as 24 hours. After a short time in either the enzyme solution or the buffer alone, however, the cells became opaque and the chromosomes tended to fragment in squashing. Apparently acetate buffer at pH 5 hardens the tissue and makes the chromosomes brittle, although it does not efface structural details.

Action of the enzyme in aqueous solutions. Trypsin in salt-free, aqueous solution effected deformation of the chromosomes in unfixed cells much more rapidly than trypsin in the presence of electrolytes. As physiological salt solution was replaced by aqueous trypsin, the cells began to swell. This was evidently an osmotic effect, since water alone evoked the same response. Membrane permeability was apparently altered in the swelling process, permitting the enzyme molecules to enter the nucleus. As a result the chromosomes of the enzyme-treated glands soon became deformed, whereas the chromosomes of the water-treated controls retained their normal appearance for longer periods of time.

Action of Trypsin on Fixed Cells

When salivary-gland cells had been fixed in 45 per cent acetic acid, the chromosomes were readily hydrolyzed by solutions of trypsin. The nature and extent of deformation produced during hydrolysis was found to depend on the composition and pH of the solvent and the duration of treatment. Whereas there was no perceptible change in the structure of the chromosomes after 24 hours' treatment with trypsin in salt-free aqueous solution at pH 6, and only slight alteration after prolonged treatment with the enzyme in acetate buffer at pH 5, marked deformation occurred within a short time when trypsin was used in acetate or phosphate buffer at pH 5.5, or in phosphate buffer in the pH range between 6 and 8.

Effect of trypsin at pH 5 and 5.5. Treatment of chromosome smears or intact glands with trypsin in acetate buffer at pH 5 for 4 hours or more did not produce any gross structural deformation of the chromosomes or affect their stainability (Fig. 7), although there was some intracapsular swelling—as evidenced by the curving outward of contiguous bands to form vesicles—in preparations treated for 16 or 24 hours (Fig. 8). When intact glands were squashed after hydrolysis in trypsin dissolved in acetate buffer at pH 5, the chromosomes tended to fragment. Since chromosomes in control preparations also fragmented when squashed, it may be assumed that acetate buffer hardens the tissue, as was suggested by its action on unfixed glands.

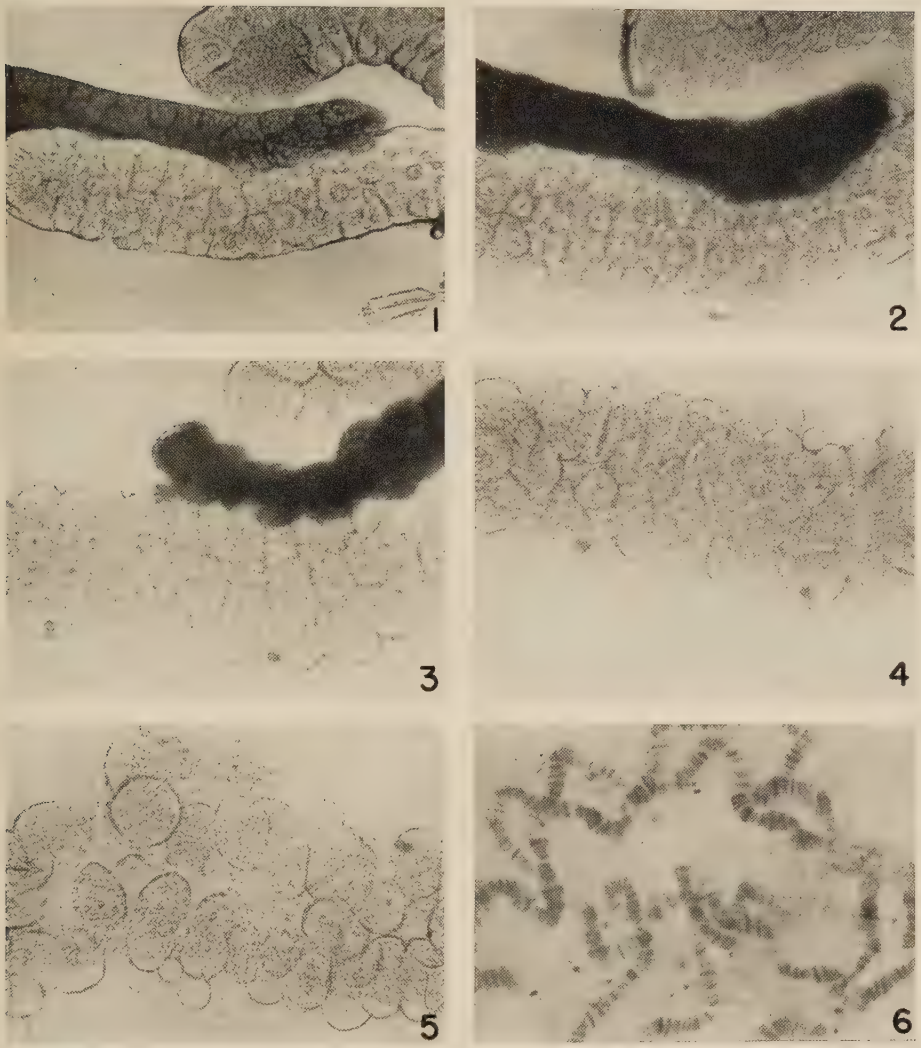
The action of trypsin in acetate buffer at pH 5.5 was more rapid and destructive, 4 minutes' treatment producing some intracapsular swelling. When stained in aceto-orcein the chromosomes appeared more fibrous than in unhydrolyzed controls, as if composed of orcein-stainable chromonemata having nodal accumulations of similar staining material at the

loci of the bands. As digestion continued, the amount of swelling increased; and after 30 minutes the chromosomes appeared as shown in Fig. 9. The moniliform contours resulted from swelling in the interband regions and along the diameters traversed by the more delicate bands while the heavier bands retained to a considerable extent their lateral continuity, although dissociated in some places into chromomere-like bodies. Prolonged treatment produced some additional lateral swelling in these banded regions, but even in squashes made from glands that had been hydrolyzed for 24 hours it was possible to recognize the limits of the individual chromosomes. The dispersed chromomeres colored brightly in orcein, indicating that these bodies had retained during hydrolysis at least part of their original content of DNA.

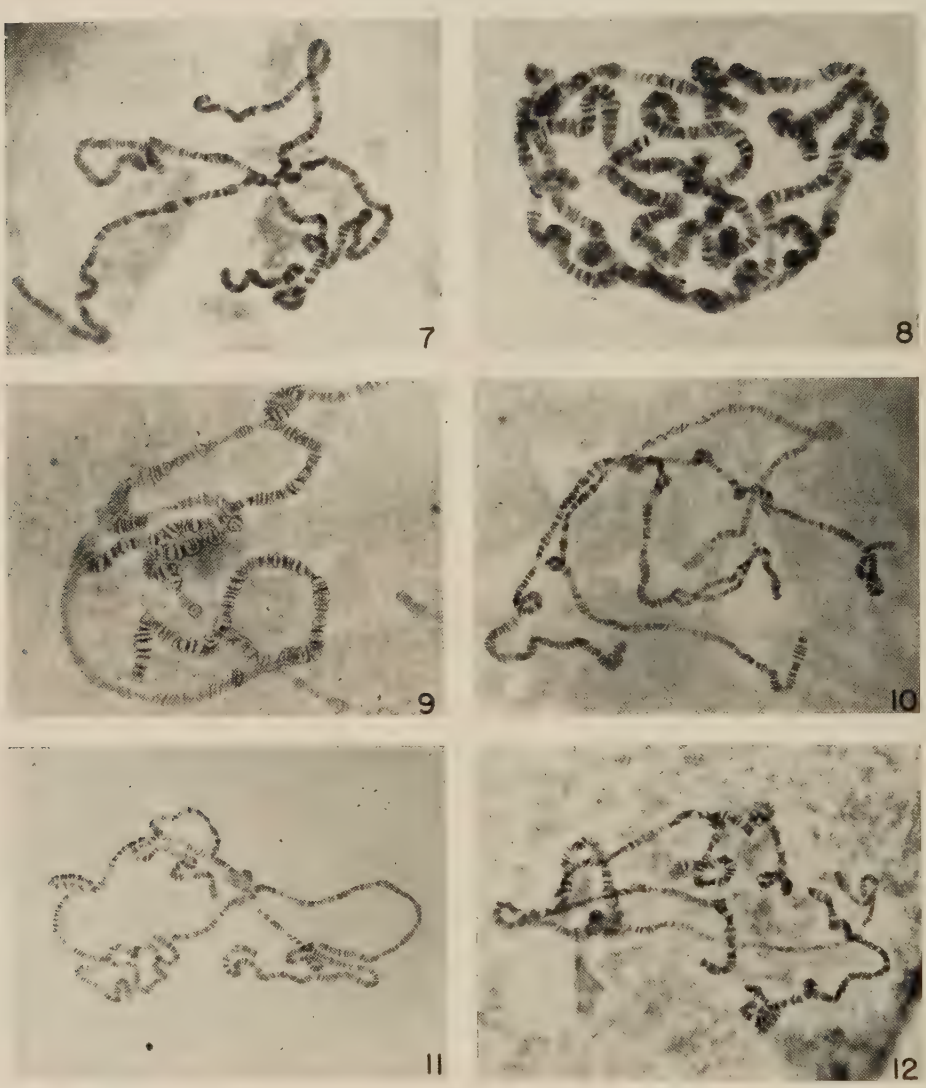
Trypsin in potassium phosphate buffer at pH 5.5 produced with slightly shorter treatment, patterns of deformation similar to those just described. Some swelling was observed, for example, in a chromosome smear that had been hydrolyzed for only 2 minutes in trypsin in phosphate buffer, as compared with the 4 minutes required for trypsin in acetate buffer. Neither the phosphate or acetate buffer in itself effected any marked alteration of chromosome structure when used for 24 hours in treating control preparations.

The role of electrolytes in producing deformation. The importance of electrolytes in effecting chromosomal deformation is clearly demonstrated by the results of experiments at pH 6. Treatment of smears or whole glands with a salt-free, aqueous solution of trypsin at this pH for as long as 24 hours did not produce any appreciable modification of chromosome structure.[1] Salivary-gland chromosomes that were hydrolyzed for 16 minutes in aqueous trypsin, rinsed in water, and stained in aceto-orcein are shown in Fig. 10. Other chromosomes that were hydrolyzed for 4 hours in aqueous trypsin, rinsed in water, and then transferred to 1 N HCl as the initial step in application of the Feulgen method, are shown in Fig. 11. Marked deformation occurred, however, when treatment with aqueous trypsin for a few minutes was followed by treatment with potassium phosphate buffer at pH 6. The chromosomes shown in Fig. 12 were hydrolyzed for 2 minutes in aqueous trypsin, rinsed thoroughly in water, and then immersed for 10 minutes in phosphate buffer before staining in orcein. Swelling and vacuolation are apparent. The amount of deformation induced by treatment with aqueous trypsin and then buffer was increased when the cells

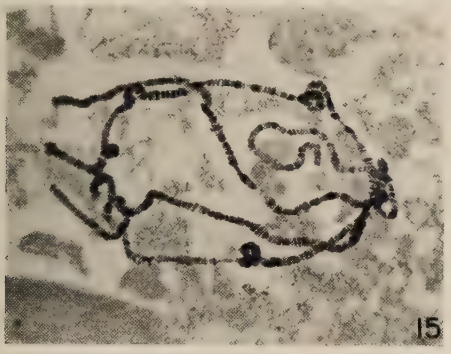
[1] Evidence has been presented (16) that a salt-free, aqueous solution of trypsin is capable of hydrolyzing substrate materials in fixed cells.

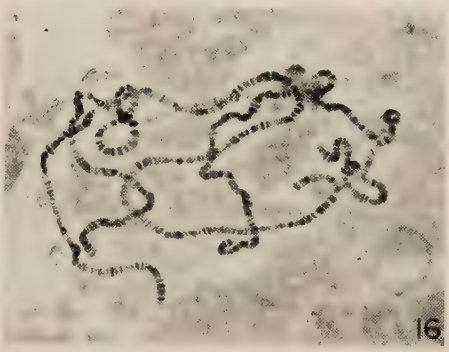


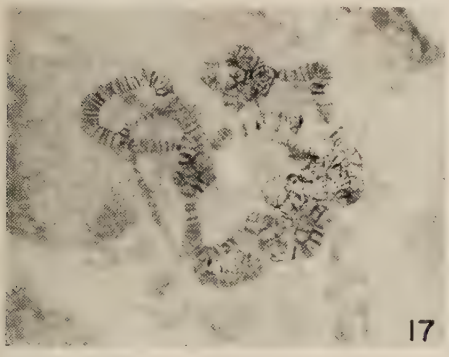
Figs. 1–6. Action of trypsin in phosphate buffer at pH 7.5 on unfixed cells.
Fig. 1. Gland in physiological salt solution. The light portions represent the salivary-gland cells; the darker strip the cells of the fat body.
Figs. 2–5. Swelling and separating of salivary-gland cells during 2 hours' treatment. Cells of the fat body also become detached in the course of treatment.
Fig. 6. Chromosomes of cells shown in Fig. 5, in aceto-orcein squash.
Magnification: figs. 1–5, 38 ×; Fig. 6, 300 ×.

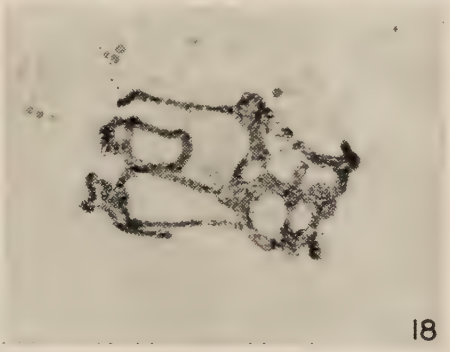


Figs. 7–8. Action on fixed cells of trypsin at pH 5 in acetate buffer.
Fig. 7. Chromosomes after 4 hours' treatment.
Fig. 8. After 24 hours' treatment; intracapsular swelling is apparent. Stain: aceto-orcein. Magnification: Fig. 7, ca. 400 ×; Fig. 8, ca. 600 ×.
Fig. 9. Treatment with trypsin in acetate buffer at pH 5.5 for 30 minutes produced marked intracapsular swelling. Aceto-orcein; ca. 400 ×.
Fig. 10. Characteristic patterns of banding in chromosomes hydrolyzed for 16 minutes in salt-free, aqueous trypsin at pH 6 and then rinsed in water before staining. Aceto-orcein; ca. 400 ×.
Fig. 11. Normal-looking chromosomes that were hydrolyzed for 4 hours in aqueous trypsin at pH 6, and then rinsed in water. Feulgen; ca. 400 ×.
Fig. 12. Chromosomes that were hydrolyzed for 2 minutes in aqueous trypsin at pH 6, rinsed in water, and treated for 10 minutes in phosphate buffer. Note vacuolation of chromosomes. Aceto-orcein; ca. 400 ×.









Figs. 13–14. Comparison of effects of trypsin-water-buffer series and trypsin-water-buffer-water series at pH 6. More deformation occurred when final rinse was given (Fig. 14) than when it was omitted (Fig. 13). Aceto-orcein; ca. 400 ×.

Figs. 15–16. Normal patterns of banding in chromosomes hydrolyzed for long periods of time in aqueous trypsin, pH 6.

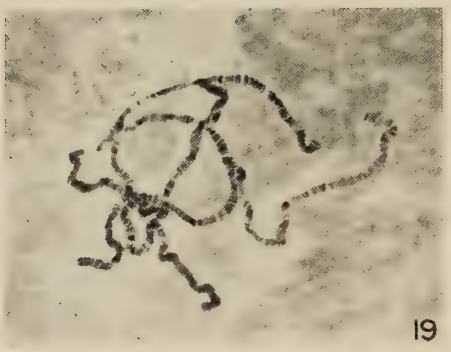
Fig. 15. 4 hours' hydrolysis followed by phosphate buffer.

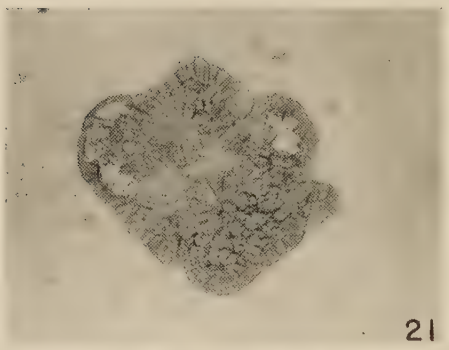
Fig. 16. 4 hours' hydrolysis followed by phosphate buffer and final water rinse. Aceto-orcein; ca. 400 ×.

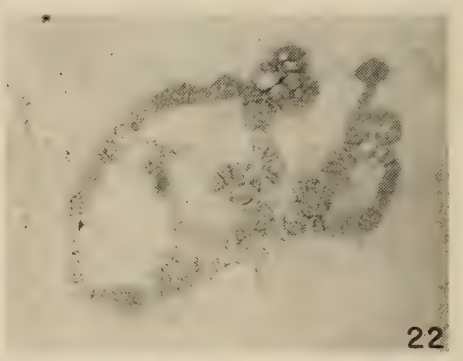
Figs. 17–18. Swelling and subsequent shrinkage of chromosomes during treatment in phosphate buffer at pH 6.5.

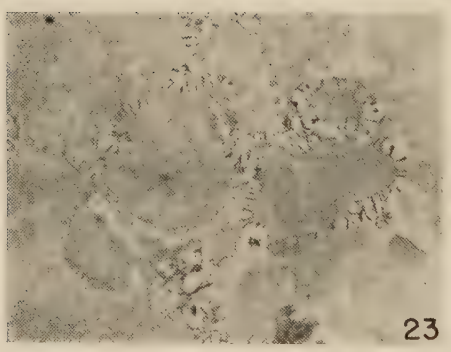
Fig. 17. Effect of 16 minutes' treatment.

Fig. 18. Effect of 2 hours' treatment. Aceto-orcein; ca. 400 ×.









Figs. 19 22. Course of deformation of salivary-gland chromosomes in trypsin in phosphate buffer at pH 7.5. Aceto-orcein; ca. 400 ×.
Figs. 23–24. Inhibition of deformation by treatment with desoxyribonuclease.
Fig. 23. Control preparations treated with $MgSO_4$, water, and trypsin in buffer at pH 7.5.
Fig. 24. Preparations treated with desoxyribonuclease, water, and trypsin in buffer at pH 7.5. Feulgen, fast-green; ca. 400 ×.

were rinsed thoroughly in water before staining (see Figs. 13 and 14). Each of the preparations from which these photographs were made was hydrolyzed for 1 minute in aqueous trypsin, rinsed in water, and treated for 10 minutes in phosphate buffer, but one of them was then transferred directly to aceto-orcein (Fig. 13) whereas the other was rinsed in water before staining (Fig. 14).

Further evidence of the importance of electrolytes in promoting swelling and chromosomal deformation is illustrated by the effects of trypsin at pH 8 when dissolved in phosphate buffer, or in water adjusted with NaOH. Swelling of chromosomes in the buffered solution began during the first minute of treatment, but there was no perceptible swelling in the NaOH-adjusted solution until after 8 to 16 minutes' treatment.

Elastic properties of the nucleoprotein gel. The amount of swelling produced by application of the trypsin-water-buffer series at pH 6 increased progressively with increasing time of hydrolysis until a maximum was reached after 8 to 16 minutes' treatment. Longer periods of hydrolysis did not promote swelling, since chromosomes that had been treated with aqueous trypsin for an hour or more, then rinsed in water and treated with buffer (or with buffer followed by water), showed normal patterns of banding (Figs. 15–16).

Similar results had been obtained when swelling of salivary-gland cells was measured after application of the trypsin-water-buffer-water series (16). The amount of swelling was found to depend on the time of hydrolysis, reaching a maximum and then falling off. This and related findings (with respect to effect of concentration and time of treatment with both enzyme and electrolytes) led to the conclusion that partially degraded nucleoproteins are capable of swelling in the presence of electrolytes, but that more completely degraded nucleoproteins no longer possess the necessary gel-like properties. In accordance with this interpretation it may be assumed that the chromosomal nucleoproteins acquire gel-like properties after short periods of hydrolysis in aqueous trypsin, but that these properties are lost as more complete degradation is effected by longer periods of hydrolysis.

The amount of detectable swelling was also found to depend on time of hydrolysis when trypsin was used in aqueous solution at pH 8, or in phosphate buffer at pH 6 or 6.5. When either of the last two solutions was used, swelling of the chromosomes occurred during the first few minutes of treatment; but contraction apparently ensued as the time of hydrolysis was increased, since preparations treated for 1 to 4 hours were more slen-

der than those treated for either 8 or 16 minutes (see Figs. 17 and 18). The shrunken chromosomes (Fig. 18) showed distorted patterns of banding, which presumably resulted from such dislocation and adhesion of the bands in swelling that they could not regain their original alignment as the swollen gel contracted.

In contrast with these findings, there was no decrease in the amount of detectable swelling with prolonged hydrolysis when trypsin was used in phosphate buffer at pH 7.3, 7.5, or 8. Hydrolysis at these pH levels for short periods of time led to pronounced and extensive swelling, which eventually culminated in rupture of the chromosomes and partial or total loss of their contents. The progress of the reaction leading to this condition is illustrated in Figs. 19 to 22, which were made from smear preparations hydrolyzed at pH 7.5 for 1, 8, 16, and 30 minutes. Swelling began after 2 minutes of treatment. As the chromosomes gradually became vesiculate the bands dissociated into chromomere-like particles. It will be noted from the photographs that no disruption of the linear continuity of the chromosomes occurred during treatment, either by fragmentation or by loss of terminal or intercalary segments. When confined within an intact nucleus, the chromosomes swelled until they pressed against one another and the enveloping nuclear membrane, which accordingly assumed a lobate contour. The nucleus then appeared to be constituted of a mass of scattered chromomeres. These observations suggest that the partially hydrolyzed nucleoproteins can swell so enormously under certain conditions that the gel frame loses its elasticity and cannot shrink back to restore the chromosomes to their original dimensions as hydrolysis continues and the nucleoproteins are degraded beyond the state conducive to swelling.

Dependence of swelling on the presence of DNA. Evidence that these gel-like properties are dependent on the presence of nucleic acid in the chromosomes was obtained in digestion experiments using nucleases. When intact salivary glands or chromosome smears were hydrolyzed in solutions of desoxyribonuclease, and were then rinsed in water and hydrolyzed in buffered solutions of trypsin (at pH 7.5 in these experiments), the chromosomes shrank but showed normal patterns of banding (Fig. 24); whereas considerable swelling and structural deformation were apparent in control preparations treated with 0.003 M $MgSO_4$, then rinsed in water and hydrolyzed in buffered trypsin (Fig. 23). Normal patterns of banding were also observed in chromosomes treated with desoxyribonuclease after hydrolysis in a salt-free, aqueous solution of trypsin, but before treatment with 0.05 M potassium phosphate buffer (a trypsin-water-DNase-water-buffer-

water series). On the other hand, considerable deformation due to swelling was observed in controls treated with a trypsin-water-$MgSO_4$-water-buffer-water series.

In contrast with the action of desoxyribonuclease, ribonuclease did not inhibit trypsin-induced swelling of the chromosomes, although the amount of swelling was less in some preparations than in controls that had not been hydrolyzed in ribonuclease. It is thus apparent that the DNA-protein complex is primarily responsible for swelling and structural deformation effected in salivary-gland chromosomes by tryptic hydrolysis. It also appears that the chromosomes are not primarily responsible for the retardation of nuclear swelling that occurs when treatment of salivary-gland cells with ribonuclease precedes treatment with the trypsin-water-buffer-water series (16).

DISCUSSION

The experimental evidence presented here indicates that the structural deformation of salivary-gland chromosomes treated with solutions of trypsin is attributable to swelling of partially degraded nucleoproteins in the presence of electrolytes. The observations and conclusions parallel those obtained in an analysis of the swelling of salivary-gland cells (16). Neither study supports the view, advanced by other workers, that crystalline trypsin causes degradation of chromosomes by digesting those bonds essential to maintenance of structural integrity. This view was derived by analogy from chemical studies of the action of trypsin on synthetic peptide derivatives (e.g., 1, 2), which indicated that the enzyme attacks linkages involving basic amino acids, and would accordingly be expected to digest all classes of proteins found in chromosomes. Analysis of the process of structural deformation has shown, however, that this expectation is not realized. Prolonged digestion of chromosomes in an enzymatically active but salt-free solution of trypsin did not alter their form or efface their characteristic patterns of banding. It was only when hydrolysis in aqueous trypsin was followed by treatment with electrolytes, or when the enzyme was used in the presence of electrolytes, that deformation occurred. Nucleic acids are involved in this process; when the DNA was removed from the chromosomes, swelling and the concomitant deformation of structure were inhibited. This finding warrants consideration of the observation of Caspersson (3) that the addition of lanthanum salts to a solution of trypsin inhibited its degradational action on salivary-gland chromosomes. Caspersson assumed that the lanthanum ions served to precipitate the nucleic acids upon

their release by digestion of the protein (. . . "die Nukleinsäure . . . im Augenblick ihres Freiwerdens sofort von den gegenwärtigen Lanthanionen gefällt wird"). We have reported elsewhere (16) that lanthanum ions prevent degradational action by inhibiting, although not completely retarding swelling of the trypsin-hydrolyzed nucleoproteins. This observation was based on studies of swelling of cells and nuclei; but chromosomes are affected in a similar manner. Caspersson also observed some swelling of the chromomeres of chromosomes treated with trypsin solutions containing lanthanum ions; but offered a different interpretation of the cause of the swelling: "Die Quellung der Chromomeren nach langdauernder Digestion muss durch Auslösen einer Komponente bedingt sein; und da das Enzym ohne Wirkung auf die Nukleinsäure ist, muss diese Komponente dass Eiweiss sein."

In summary, the experiments of the present study have shown that the hydrolytic action of trypsin alters the pattern of association of proteins and nucleic acid, and that the partially degraded materials acquire in the presence of electrolytes a gel-like property that permits them to swell. Since an analysis of the swelling reaction has been presented elsewhere (16), further discussion will be restricted to consideration of the observations described above with relation to problems of chromosome morphology.

One of these problems concerns the pattern of association of proteins and nucleic acids. It has been inferred that protein alone suffices to maintain the linear continuity of the chromosome (see, for example, (6, 8, 9, 25)) and the observation that solutions of trypsin cause disintegration of the chromosomes seemed to lend support to this interpretation. Since, on the contrary, treatment with nucleases does not impair structural integrity, it was assumed that nucleic acids are transitory structural components, which can become attached at specific loci on the permanent polypeptid chain or released therefrom during the cycle of mitosis. The validity of this distinction, insofar as it rests on evidence of the disintegrative action of trypsin, is undermined by the discovery that trypsin *per se* does not effect dissolution of the chromosomes or impair their structural continuity. Dissolution can be produced, however, by successive treatments with certain combinations of enzymes, or enzymes and chemicals. Experiments involving such treatments have led to the conclusion that the chromosome represents a complex fabric in which ribose and desoxyribose nucleic acids are intimately associated with each other and with histones and nonhistone proteins (14).

Inferences about the location of different types of proteins in the chro-

mosome have also been drawn from comparisons of the effects produced by trypsin and pepsin. Since solutions of pepsin had been observed merely to cause shrinkage, in contrast with the degradational action of solutions of trypsin, it was assumed that the action of pepsin served to reveal the presence of a matrix protein, nonhistone in nature and unessential to the fundamental construction of the salivary-gland chromosome (19). Although there is now abundant chemical and cytochemical evidence that chromosomes contain both histones and nonhistone proteins, this distinction is not clearly demonstrated by comparing the action of trypsin and pepsin. In the light of the evidence reviewed in preceding discussion, it became apparent that comparisons of this type should be made by determining the gel-like properties of chromosomal materials under similar conditions of pH and associated electrolytes. Accordingly, efforts were made to test the action of trypsin and pepsin under a wide variety of experimental conditions. When this was done it was found that either of the enzymes could cause swelling or shrinkage, depending on the method of treatment. The details of the experiments will be published elsewhere, but they have clearly shown that the difference between shrinking and swelling is not determined by the selective action of either enzyme on any specific class of protein. Apparently trypsin and pepsin (and also chymotrypsin, which we have studied) attack peptide linkages in both histones and nonhistone proteins (14, 21). In the course of such cytochemical studies, in which treatments were given with two or more enzymes, it was observed that pepsin often was far more drastic than trypsin in its action on cellular substrates. Such results might have been anticipated on the basis of chemical studies in which pepsin appeared to have a considerably wider range of specificity than trypsin (7, 24).

Studies of the chromosomes during the swelling process have also shed new light on the organization of the salivary-gland chromosomes of *Drosophila.* The appearance of bands along these chromosomes depends, according to the most widely accepted interpretation, on the close approximation or confluence of homologous chromomeres of parallel-lying chromonemata. Observations of the swollen chromosomes confirmed this interpretation. Bands that appeared continuous in the untreated chromosome often appeared as a series of separated chromomeres in the hydrolyzed preparations. In some of these preparations the chromonemata were clearly defined, extending along the chromosome and connecting the chromomeres of adjacent bands. Details of these studies will be published in another article.

SUMMARY

The action of solutions of purified, crystalline trypsin at various pH's on unfixed and fixed salivary-gland chromosomes of *Drosophila melanogaster* is described and illustrated. Chromosomes of glands transferred directly from physiological salt solution to buffered solutions of trypsin showed no evidence of enzymatic degradation during the first few hours of treatment. Chromosomes of fixed cells were readily hydrolyzed by trypsin dissolved in either water or buffer. Structural deformation did not occur during hydrolysis in aqueous trypsin, but was induced by subsequent treatment with phosphate buffer, or during the course of hydrolysis in buffered solutions. The amount of deformation was found to depend on the pH and duration of the treatment. Structural deformation is attributed to the swelling of partially degraded nucleoproteins in the presence of electrolytes, since removal of desoxyribonucleic acid from the chromosomes inhibited swelling. The application of the findings to problems of structural organization is discussed.

The author desires to express his appreciation to Dr. Margaret McDonald for preparing and assaying the enzymes used in this study, and for critical inspection of the manuscript. He also wishes to thank Miss Janice Larrabee for assistance in the technical aspects of the work.

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THE ULTRASTRUCTURE OF THE PROXIMAL CONVOLUTED TUBULES OF THE MOUSE KIDNEY AS REVEALED BY HIGH RESOLUTION ELECTRON MICROSCOPY

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Originally with the aim of studying the ultrastructure of mitochondria the tubular cells of the mouse kidney were analyzed through electron microscopy of ultrathin sections. As several new observations regarding the organization of the tubular cell cytoplasm were made we have extended the analysis to the ultrastructure of the cells of the proximal convoluted tubules in general.

Systems of double-edged membranes organized as cell membrane, intracellular membranes, nuclear membrane and outer and inner mitochondria membranes have been revealed and made accessible to measurements. The analysis of the "brush border" has also been started.

The analysis is being continued in order to try to reveal even more minute structures the existence of which has been indicated in our micrographs but not yet in a sufficiently obvious way to be adequately described.

An extensive *light microscopic analysis* of the tubular cells of the kidneys from mouse, rat, rabbit and guinea-pig was published in 1944 by Sjöstrand (18). The literature up to this year was also critically reviewed. The artefacts produced through fixation were pointed out and in order to reduce fixation artefacts as much as possible, the tissue was fixed by means of freeze-drying and only such specimens were selected for the analysis which, within the well preserved areas, did not show light optically resolved ice crystal vacuoles.

The differentiation of the nephron in parts exhibiting structurally obvious differences could be developed further, due to the combined use of fluorescence microscopy and ordinary light microscopy. The effect of different functional states on the structure also was tested.

The detailed knowledge of the common fixation artefacts and the rather extensive and complicated differentiation of the nephron are obvious prerequisites for an electron microscopic analysis where the small field renders the orientation even more difficult than in light microscopy.

The basic facts regarding the light microscopy of the tubular cells will be

mentioned in connection with the discussion of the electron microscopic observations. For a more detailed account the reader is referred to the monograph just mentioned.

Electron microscopic studies of the kidney cells have been published by Dalton, Kahler, Striebich and Lloyd (4), Pease and Baker (15), Dalton, Kahler and Lloyd (3) and Dalton (2).

Dalton *et al.* perfused a mixture of osmium tetroxide, lanthanumsulfate and potassium bichromate through the kidney for fixation, Pease and Baker used 2 per cent osmium tetroxide without perfusion instead.

Pease and Baker were not able to observe the cell membranes completely separating the cell territories. In connection with the mitochondria they observed "cytoplasmic condensations" forming tubular sheaths surrounding the mitochondria. These sheaths should attach to the membrane at the basal portion of the cell. They considered that "in life the sheaths may have more the character of a gel layer than that of definite membranes."

Dalton (2) observed interdigitating cell membranes which he interpreted as corresponding to the complicated interdigitations of the cell boundaries. In addition he described "basally located intercellular filament-like structures approximately 0.05 μ in width," which according to the author is somewhat thinner than the cell membranes. The interdigitating cell membranes measure 0.18 μ in thickness according to Dalton *et al.* (4).

Pease and Baker (15) described the "brush border" consisting of vast numbers of relatively long thin projections "tremendously increasing the surface exposed to the lumen of the nephron." Dalton *et al.* (4) described the "brush border" as filaments 0.07 μ in diameter. The mitochondria have appeared too dense to exhibit any defined internal structures in these investigations.

MATERIAL AND METHODS

The adult white mice used as experimental animals were killed by decapitation. 0.3–0.5 mm thick slices of the kidney tissue were cut with a razor blade knife and fixed in 1 per cent osmium tetroxide buffered to pH 7.2 with acetate-veronal buffer.[1] The tissue was embedded in n-butylmethacrylate principally according to Newman, Borysko and Swerdlow (14).

The Spencer rotary microtome model 820 was used for sectioning with the standard adapter to reduce the feed and with modifications similar to the ones recommended by Hillier and Gettner (12, 13). For further details the reader is referred

[1] We feel very much indebted to Dr. K. Porter and Dr. G. E. Pallade for their kindness in giving the necessary information regarding the fixation technique used in this investigation. During the printing of this paper the paper by G. E. Pallade describing the fixation technique in *J. Exptl. Med.* **95**, 285 (1952) has become known to us.

to the paper by Sjöstrand (19). Especially polished Schick razor blades mounted in a curved position were used as knives (19, 20). The sections were collected on a liquid surface, using a trough similar to the one originally described by Gettner and Hillier (8). As liquid 20–30 per cent ethyl alcohol was used.

The sections were floated onto the specimen grids covered with a thin formvar film. They were examined in the electron microscope without dissolving the methacrylate and without subliming the methacrylate by subjecting them to a high beam intensity. On the contrary the examination was performed at a low beam intensity.

An RCA EMU 2 c electron microscope was used with compensated standard objective pole piece, with standard objective aperture, with three apertures in the projector lens (according to Hillier's recommendations) and reduced condensor lens aperture.

The magnifications were calibrated by using chromium shadowed replicas of an 1 152 lines per mm diffraction grating and Dow latex particles, 58 G lot 3 584. For further details see Sjöstrand (19).

The resolution of the micrographs stated is based on measurements which have determined the minimum distance between image points which are resolved.

RESULTS

The proximal convoluted tubules is differentiated in four different parts (18). The most distal of these, the *pars recta*, exhibits the most obvious differences especially with regard to the structure of the "brush border." This part has not yet been sufficiently analysed and will not be included in the following description.

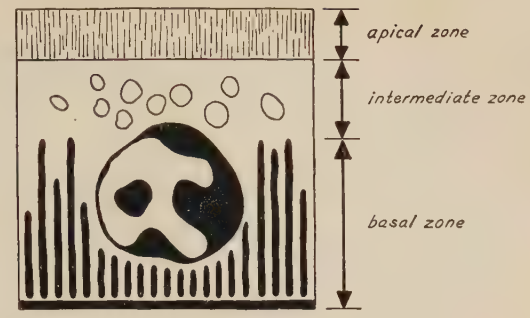
The present analysis has been performed at a resolution of 35 Å. It has been possible to study the tubular cells in very well preserved conditions without artificial vacuolization or obvious artificial condensations of the cytoplasm. The basic structures have corresponded to what regularly can be observed with the light microscope on well preserved specimens.

General description. The cells of the proximal convoluted tubules are organized according to a rather regular pattern. A basement membrane surrounds the tubule. When fixed under normal conditions the lumen of the tubule is collapsed except for the *pars recta*. A basal, main part of the cells contains the nucleus and the mitochondria which are rod-shaped and oriented almost perpendicularly to the basement membrane.

The most apical part of the cells is characteristically organized as the so called "brush border." In between the "brush border" and the apical ends of the mitochondria there is another cell zone characterized by vacuoles, granules and extensions from the "brush border" structure.

These three cell zones will be referred to as the *basal*, the *apical* and the *intermediate zones*. (See the schematic drawing, Textfig. 1.)

The ground cytoplasm with intracellular cytoplasmic membranes. Within the basal cell zone the ground cytoplasm is divided into open compartments through well defined intracellular double membranes (Fig. 1–2 a–b). These



Textfig. 1. Schematic drawing of the structure of the proximal convoluted tubule cells as revealed by the light microscope.

membranes are localized between the mitochondria and enclose compartments which in most cases contain several mitochondria arranged in rows.

The compartments are closed at the basal end where the intracellular membranes reach to a distance of about 200 Å from the basement membrane and here form rounded bottoms of the elongated compartments (Fig. 3). Fig. 5 shows a tangential section through the basal part of a tubular cell with the intracellular compartments cut almost transversally. At the border between the basal and intermediate zones these compartments are open.

This dividing of the cytoplasm in a series of compartments seems to be more elaborate in some cells than in others. If this reflects an additional differentiation of the different parts of the proximal convoluted tubule or merely depends upon varying preservation is still uncertain.

The membranes appear as double membranes. The two constituent single membranes exhibit a marked contrast and are separated by a light space. The mean distance between the two membranes when measured as the distance between the centers of the cross cut membranes is 190 ± 6 Å. The total thickness of the double membranes is 270 ± 7 Å (Table I). Each mean value is based on 600 measurements performed on 25 different intracellular membranes. As is seen from Table I there is an irregular distribution of the values with two main groups. This might well depend upon differences regarding the fixation. The mean thickness of the individual membranes can be calculated to about 80 Å and the mean width of the space in between the two membranes to about 110 Å.

In the ground cytoplasm there are groups of rather dense dots, each dot measuring about 100 Å in diameter. These dots are especially numerous in the basal cell zone in between the mitochondria. Each such dot is composed of a few granules with a diameter of about 40 Å. In addition to these regularly occurring dots there are indications of more or less irregular very thin

TABLE I

The dimensions of the intracellular cytoplasmic membranes.

Each figure in the second and third column represents the mean value of 24 measurements.

Membrane number	Total thickness Å	Distance between centers of the constituent single membranes Å	Calculated thickness of the single membranes Å	Calculated height of the space between the single membranes Å
1	337	244	93	151
2	373	270	103	167
3	277	208	69	139
4	270	202	68	134
5	221	150	71	79
6	234	154	80	74
7	210	147	63	84
8	261	202	59	143
9	248	171	77	94
10	239	167	72	95
11	265	177	88	89
12	223	160	63	97
13	259	172	87	85
14	282	177	105	72
15	274	171	103	68
16	267	174	93	81
17	280	186	94	92
18	304	190	114	76
19	228	154	74	80
20	247	190	57	133
21	271	203	68	135
22	294	219	75	144
23	286	208	78	130
24	285	203	82	121
25	270	197	73	124
Mean	270 ± 7[1]	190 ± 6[2]	80	110

membranes or fibrils distributed in the ground cytoplasm, sometimes forming an irregular network (Fig. 2 a). These observations are too few, however, to justify too much attention for the moment.

The basement membrane. The basement membrane forms a complete sheath around the tubule between the tubular cell cytoplasm and the intersti-

[1] The coefficient of variation 36 per cent.
[2] The coefficient of variation 31 per cent.

TEXT TO FIGURES

Fig. 1. General survey picture of the different zones of the proximal convoluted tubule from mouse kidney. At the bottom the basement membrane separated by an about 200 Å wide space from the plasma membrane. To the right the nucleus. In the basal cell zone mitochondria and intracellular cytoplasmic membranes. In the upper part the "brush border." In the intermediate cell zone vacuoles and cross cut and obliquely cut extensions from the "brush border" structure. Magnification 24 000 ×.

Fig. 2 a. The basal cell zone of a proximal convoluted tubule cell. Mitochondria with outer and inner double membranes and dark areas are seen. Between the mitochondria intracellular cytoplasmic membranes. The ground substance of the cytoplasm exhibits a rather regular granular appearance and in some areas fibrils forming networks. Magnification 47 000 ×.

Fig. 2 b. Enlarged detail from Fig. 2 a. This figure represents a high resolution picture of the mitochondria double membranes. Magnification 137 000 ×.

Fig. 3. The basal zones of some proximal convoluted tubule cells. The basement membranes are shown and in the left tubule many intracellular cytoplasmic membranes are seen forming the boundary of the cytoplasm towards the basement membrane. Magnification 27 000 ×.

Fig. 4. The "brush border" structure cut transversally and longitudinally, survey picture. Magnification 18 000 ×.

Fig. 5. Tangential section through the basal part of a proximal convoluted tubule cell showing cross cut intracellular compartments separated by double membranes. Magnification 31 000 ×.

Fig. 6. The apical parts of some proximal convoluted tubule cells with the "brush border" structure, vacuoles, mitochondria and at the bottom a part of a nucleus. At the right side a double membrane runs all the way to the "brush border." It constitutes the cell membranes between two adjacent cell territories. Magnification 47 000 ×.

Fig. 7. Detail of a proximal convoluted tubule cell at the border between the basal and the intermediate cell zones with mitochondria, a granulum and at the right lower corner a part of a nucleus. The granulum is here located between the mitochondria and the nucleus in the centre of the picture. Double membranes may be seen in the ground substance of the cytoplasm and in the mitochondria, around the nucleus and around the granulum. In the ground substance of the cytoplasm groups of minute granules are seen consisting of groups of a few smaller granules. Magnification 90 000 ×.

Fig. 8. The "brush border" and the intermediate cell zone with granules and vacuoles. To the left the lumen of the tubule, to the right a granule with an internal extremely dense particle (the contrast has been decreased at the printing in order not to obtain a general blacking all around this granule). Cross cut and obliquely cut extensions from the "brush border" are seen and groups of minute granules are scattered in the ground substance of the cytoplasm. Magnification 46 000 ×.

Fig. 9. Cross cut "brush border" showing the dense linings of the "brush border" ducts and the opacity of different parts of the "brush border." Magnification 51 000 ×.

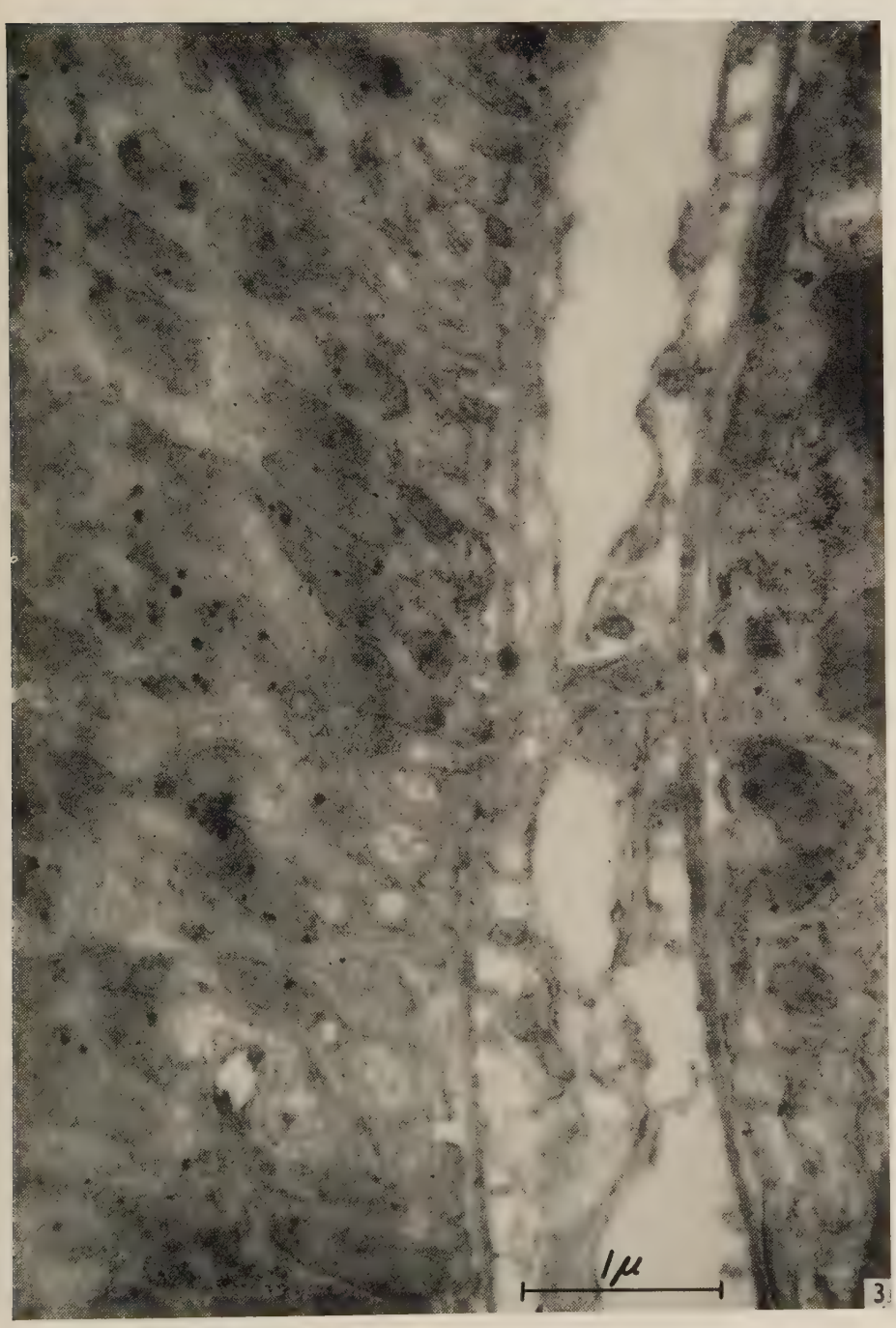
Fig. 10. Light microscopic picture of proximal convoluted tubules of mouse kidney fixed by freeze-drying, stained with Heidenhains iron-hematoxylin. Notice the optically homogeneous ground substance of the cytoplasm and the homogeneous "brush border", the intensely stained rod-shaped mitochondria and the light vacuoles in the intermediate cell zone. Magnification 1 460 ×.

Fig. 11. Fluorescence microscopic picture of frozen-dried mouse kidney. Paraffin embedding, the paraffin has not been removed. The tubules with the bright granules are proximal convoluted tubules. The fluorescence light of the granules is yellow and of the mitochondria is blue to bluish green. The amount of granules, the colour of their fluorescence and the fluorescence of the mitochondria differs in different parts of the proximal convoluted tubule. The fluorescent granules appear as vacuoles in Fig. 10.

1μ
1

1μ
2a

0.1μ
2b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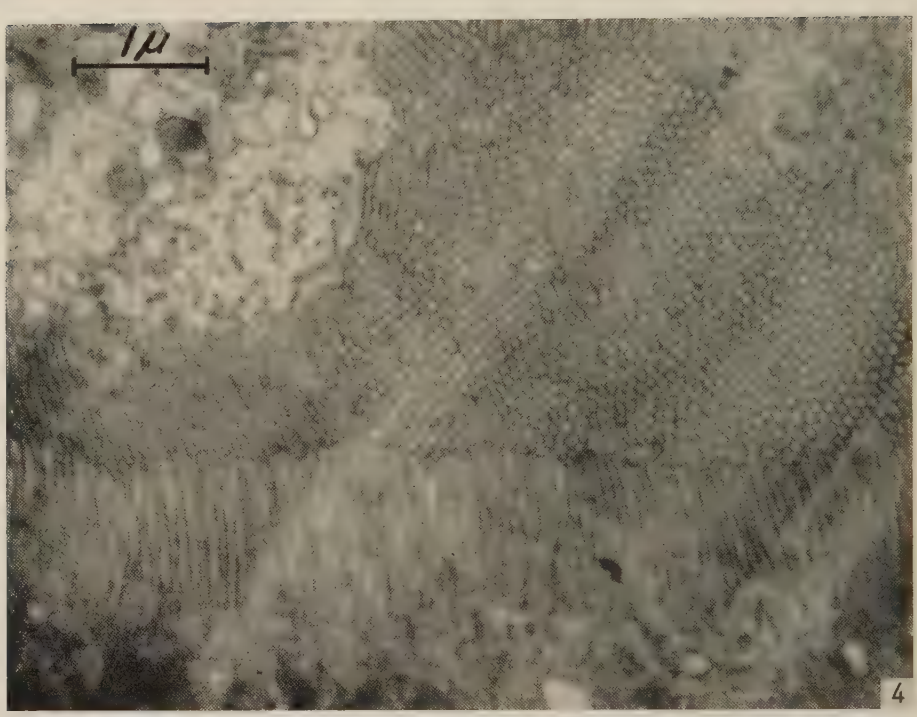
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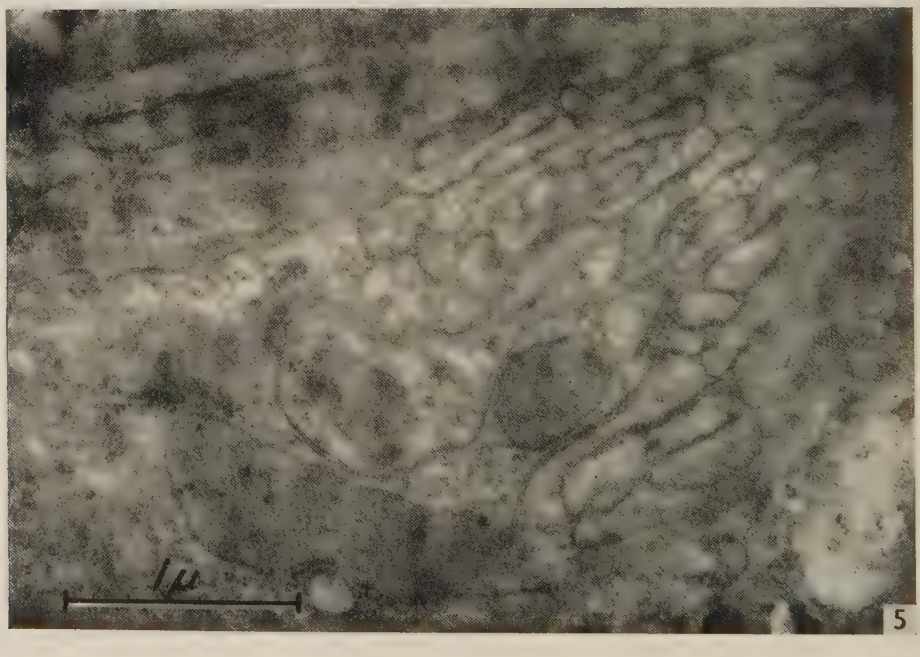
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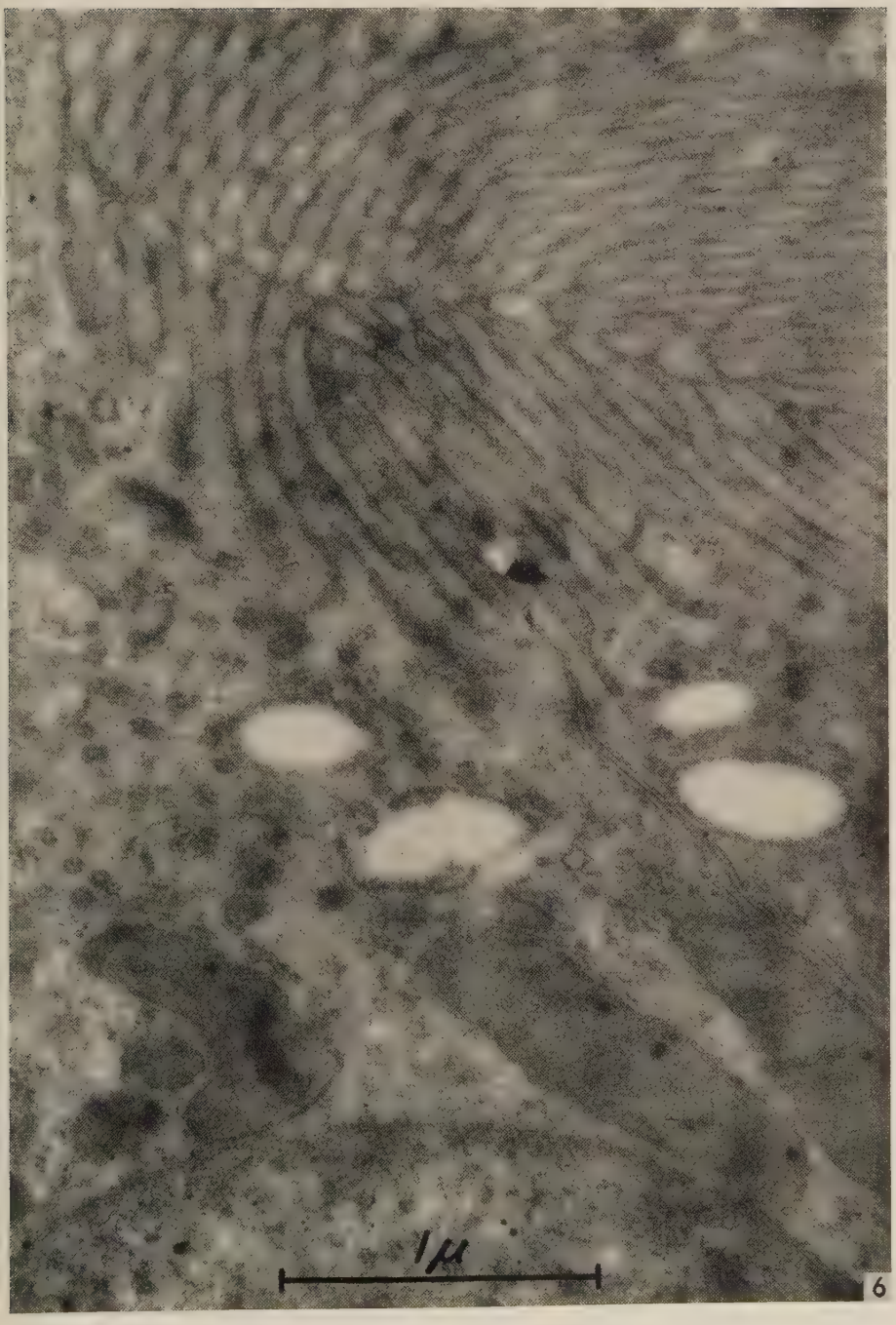
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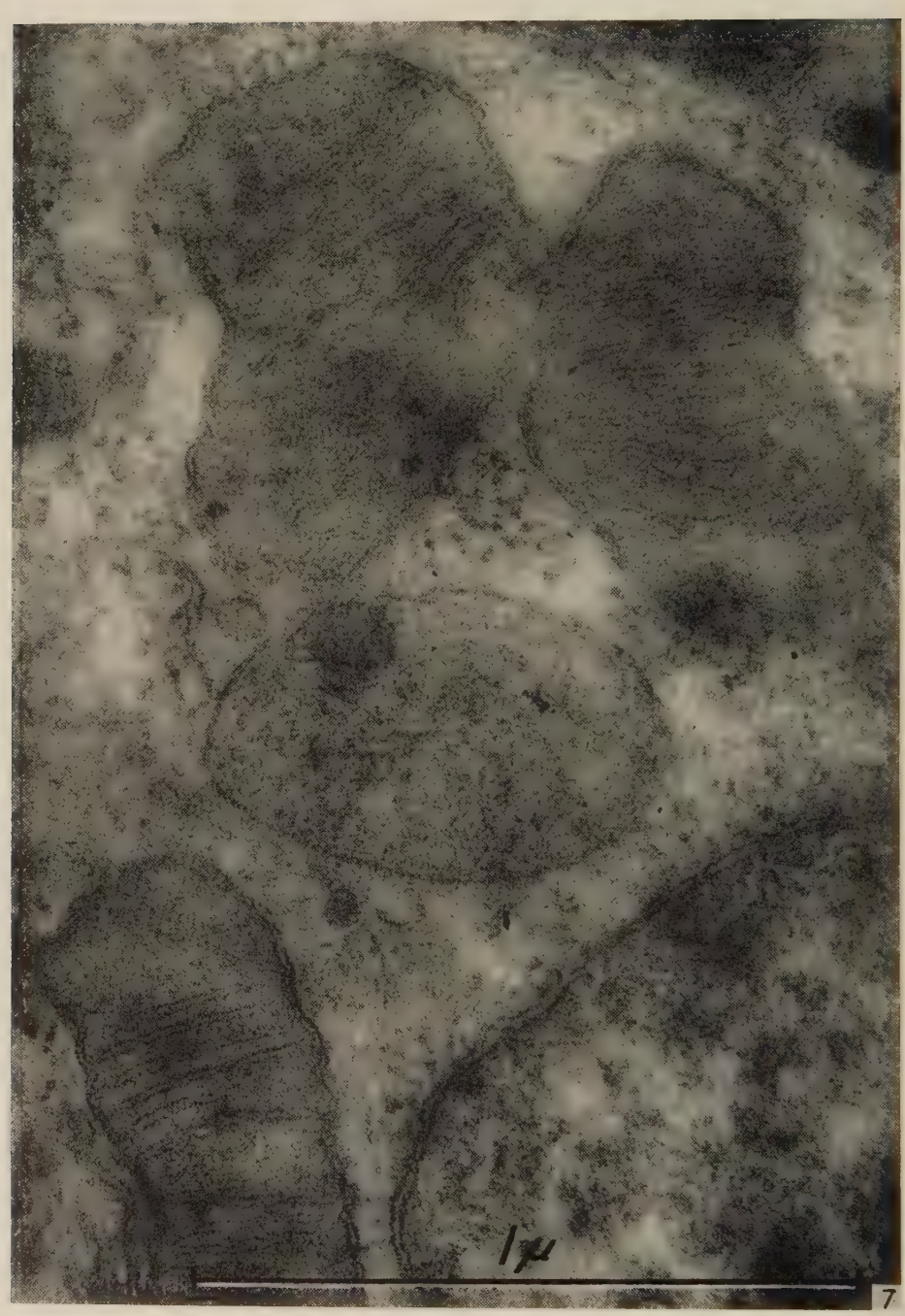
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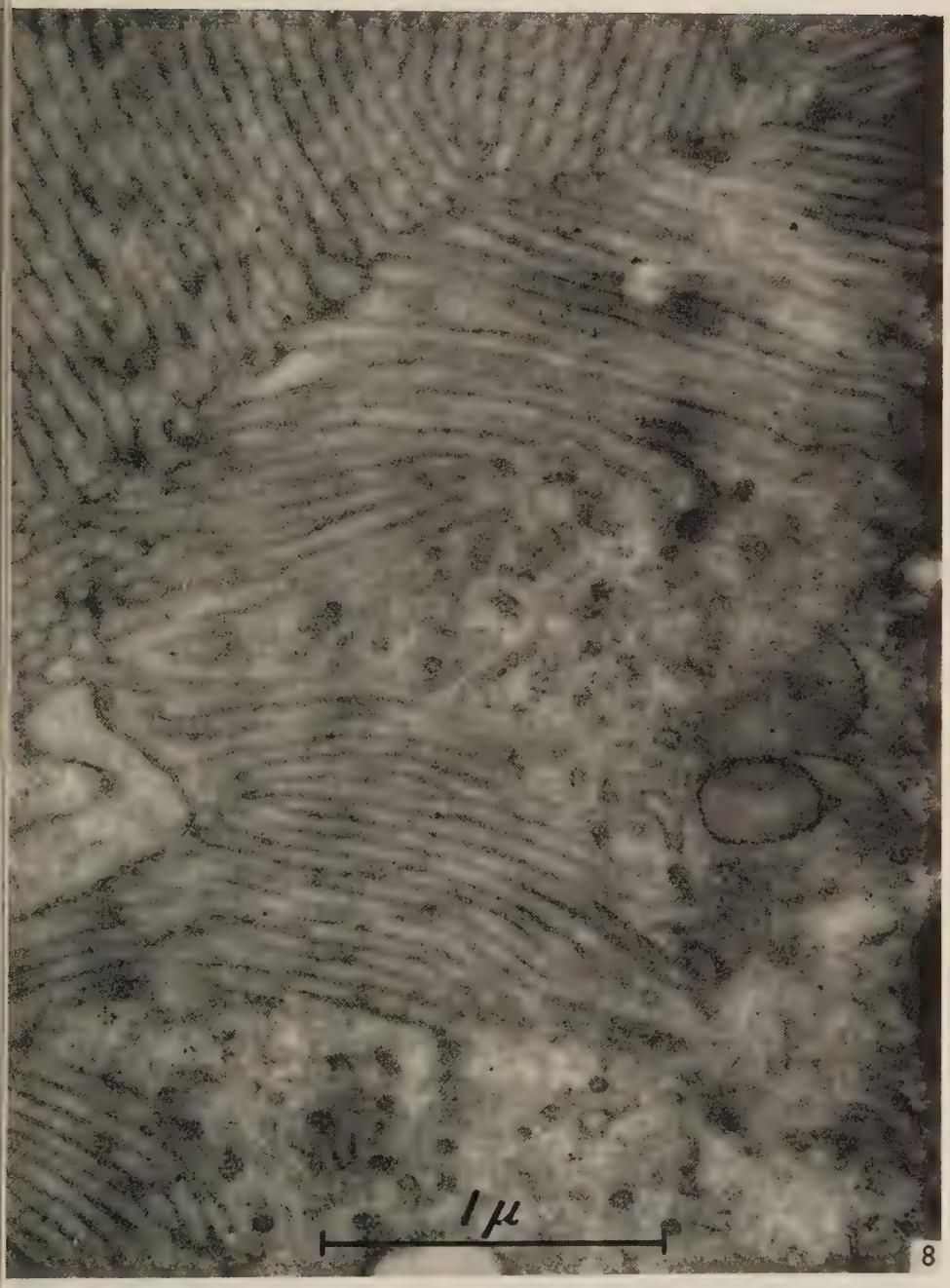
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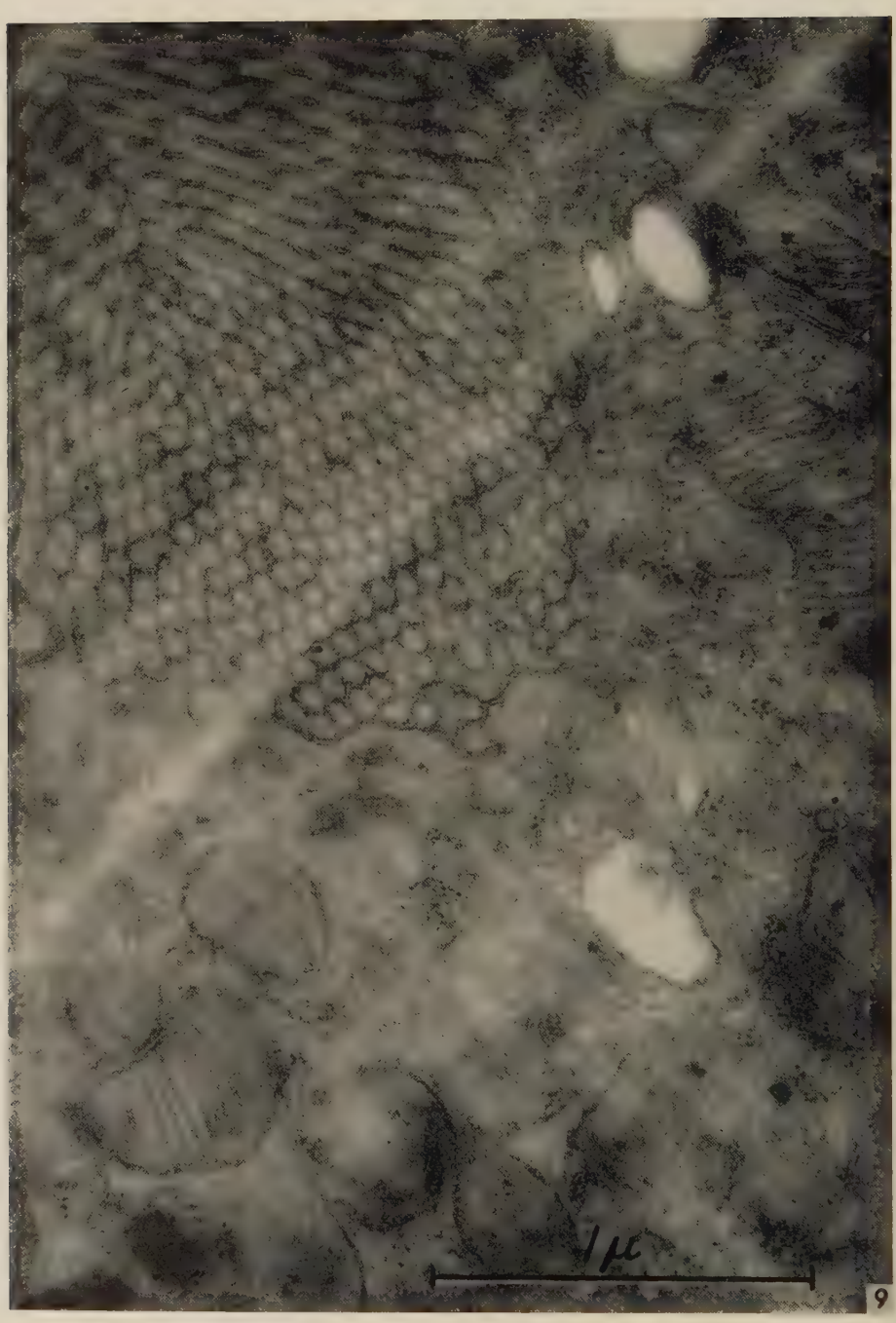
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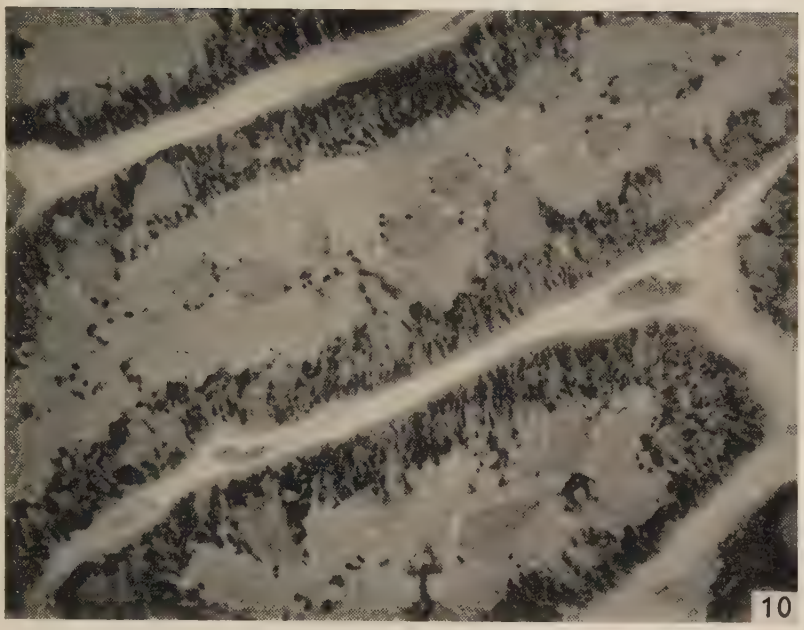

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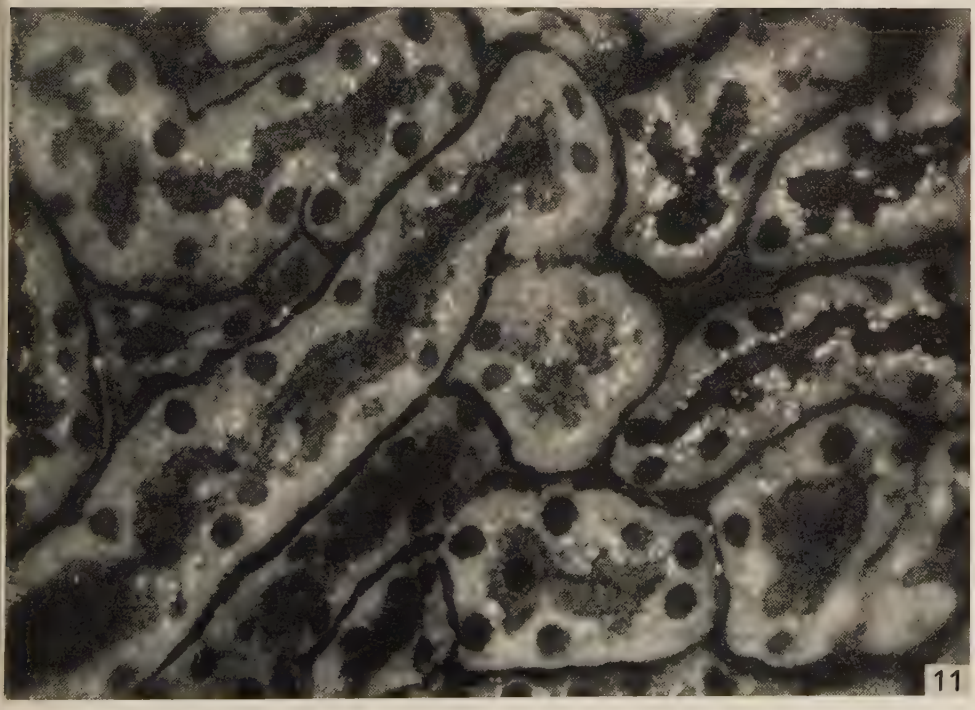

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tial cell elements. These latter cells cover the basement membrane completely.

It has not been possible to observe any regular structural pattern in the basement membrane. Its thickness varies considerably to a great extent due to different angles of sectioning. The lowest values obtained are 0.05 to 0.1 μ.

The cell membranes. Towards the basement membrane the cytoplasm appears somewhat condensed as judged from the greater contrast (Fig. 1) forming a well marked and distinct surface, facing the basement membrane and separated from the latter by a space about 200 Å wide. In those areas where the intracellular cytoplasmic membranes reach the vicinity of the basement membrane they replace the cell membrane as a border towards this minute space between the cytoplasm and the basement membrane.

The cell territories within the tubules are separated by double membranes (Fig. 6). These double membranes are similar to the intracellular membranes but in contrast to these membranes they reach the apical cell zone to connect with the "brush border" structure. At the basement membrane they do not seem to behave differently to the intracellular membranes.

In specimens where the membranes are well preserved all cell territories are completely separated by such double membranes.

The thickness of the cell membranes is 280 Å. This figure is based on 72 measurements on three different cell membranes. The distance between the centers of the constituent single membranes of the double membranes has been measured to 220 Å. The thickness of each individual membrane, therefore, would be 60 Å and the width of the space in between the single membranes would be 160 Å. The two last figures differ considerably from the corresponding dimensions of the intracellular membranes but the observations are too few to allow any conclusions from this discrepancy.

The mitochondria. The mitochondria are rod-shaped and arranged in rows within the intracellular compartments of the basal cell zone. The length of these rods shows a pronounced variability and it has not yet been possible to reach a definite opinion regarding their real length as their orientation seems sufficiently irregular to make it difficult in such thin sections to secure a longitudinal section through the whole length of the mitochondria.

The mitochondria show a well differentiated and easily defined structure (Figs. 1, 2 a–b, 6, 7 and 9). Each mitochondrion is surrounded by an outer limiting double membrane. In addition there is in the interior of the mitochondria a system of internal double membranes oriented chiefly transversally to the long axis of the mitochondria.

The outer as well as the inner double membranes are well defined with the two individual membranes of the double membranes mainly running parallel but with sufficient lack of parallelism to demonstrate a certain individuality.

The total thickness of the outer double membranes is 160 ± 2 Å. The distance between the centers of the constituent single membranes of the double membranes is 115 ± 2 Å (Table II). Each mean value is based on 600 measurements on 25 different mitochondria. The thickness of the single membranes can be calculated from these figures to 45 Å and the height of the space between the single membranes to 70 Å.

The inner membranes of the mitochondria are arranged chiefly mutually parallel and may extend to or be separated by a distance of 100–200 Å from the outer membrane. At the edge of the perpendicularly cut inner double membranes the two constituent membranes are connected, the space in between the membranes being closed. The dimensions of the inner membranes are for the total thickness 160 ± 2 Å, and for the distance between the centers of the single membranes 120 ± 1 Å (Table III). Each mean value is based on 600 measurements on 25 different mitochondria. The calculated thickness of the single membranes will be 45 Å and for the height of the space in between the single membranes 70 Å. None of these figures show any significant difference from the corresponding figures for the outer membranes. The outer and inner mitochondria membranes, seem to be morphologically identical.

The inner double membranes are separated by 170–470 Å wide spaces. Scattered along the mitochondria there appear dark areas due to the localization of a material with high scattering power, probably reduced osmium. The size of these areas varies from 200 Å to 700 Å. They appear as contaminations but their localization is restricted to the mitochondria and the inner double membranes of the mitochondria exhibit a curved, diverging course on both sides of these particles. They, therefore, obviously are structural elements of the mitochondria (Figs. 2 a–b, 6, 9).

The ground substance of the mitochondria has not shown any obvious structure that could be revealed at the resolution of these micrographs.

The "brush border." The "brush border" constitutes a cell zone characterized by a high degree of organization according to a rather regular pattern. When studied under the conditions described here with a collapsed tubular lumen the surface of the cells facing the lumen is smooth and the cells are bordered by a single membrane (Fig. 8).

From this surface a great number of tightly packed tubes or ducts of

TABLE II

The dimensions of the outer mitochondria double membranes.
Each figure in the second and third column are mean values of 24 measurements.

Mitochondrion number	Total thickness Å	Distance between centers of the constituent single membranes Å	Calculated thickness of the single membranes Å	Calculated height of the space between the single membranes Å
1	139	99	40	59
2	155	117	38	79
3	157	110	47	63
4	170	126	44	82
5	160	117	43	74
6	152	106	46	60
7	154	109	45	64
8	156	112	44	68
9	155	114	41	73
10	165	122	43	79
11	167	128	39	89
12	151	101	50	51
13	152	114	38	76
14	155	113	42	71
15	163	120	43	77
16	164	125	39	86
17	159	115	44	71
18	166	127	39	88
19	152	106	46	60
20	157	107	50	57
21	162	113	49	64
22	161	108	53	55
23	194	132	62	70
24	190	132	68	64
25	173	118	55	63
Mean	160 ± 2[1]	116 ± 2[2]	45	70

rather uniform dimensions extend towards the interior of the cell body. These ducts are closed towards the lumen and at their base. Here the walls of the ducts continue in rod-shaped extensions which exhibit an irregularly curved course within the intermediate cell zone. Here they are observed as small granules when cut across or as short rods when cut obliquely. It is not possible, of course, to establish that all the small granules observed within this cell zone represent cross-cut extensions from the "brush border" elements.

[1] The coefficient of variation 12 per cent.
[2] The coefficient of variation 9 per cent.

TABLE III

The dimensions of the inner mitochondria double membranes.
Each figure in the second and third column are mean values of 24 measurements.

Mitochondrion number	Total thickness Å	Distance between centers of the constituent single membranes Å	Calculated thickness of the single membranes Å	Calculated height of the space between the single membranes Å
1	140	105	35	70
2	164	120	44	76
3	158	111	47	64
4	143	106	37	69
5	156	116	40	76
6	156	114	42	72
7	162	119	43	76
8	183	128	55	73
9	186	128	58	70
10	163	127	35	92
11	162	119	43	76
12	171	129	42	87
13	170	119	51	68
14	169	129	40	89
15	167	125	42	83
16	164	125	39	86
17	161	117	44	73
18	159	112	47	65
19	162	121	41	80
20	165	117	48	69
21	162	121	41	80
22	162	116	46	70
23	161	117	44	73
24	158	111	47	64
25	167	108	59	49
Mean	163±2[1]	118±1[2]	45	75

The ducts are lined by a dense surface structure. Between two adjacent ducts these surface structures form a double-edged wall, a less dense zone separating the denser surface structures of adjacent duct walls. When cut across, the "brush border" appears like a honey-comb (Fig. 9). The ducts, however, exhibit a circular and not hexagonal cross section.

The density within the lumen of the ducts corresponds to the density of the ground substance of the cytoplasm and it seems reasonable to assume that the ducts are not hollow tubes but cytoplasmic compartments lined by

[1] The coefficient of variation 10 per cent.
[2] The coefficient of variation 7 per cent.

a denser surface structure. The inner diameter of the "brush border" ducts is about 600 Å with some variation presumably due to differences in the different parts of the proximal convoluted tubules. The thickness of the walls separating adjacent duct lumens is about 150 Å. The length of the ducts, the basal extensions not included, is 1–1.5 μ. The rod-shaped extensions at the base of the "brush border" measure about 600 Å in diameter. Many of them show a less dense center which has been interpreted as a thin basal extension of the duct lumen.

It has been demonstrated by light microscopy, also on frozen-dried kidney tissue, that there are cells in the proximal convoluted tubule, the protruding apical surface of which is not completely covered by a "brush border." These cells have been called "dome cells." They regularly appear in this material and according to the electron micrographs they may best be described as cells with incomplete "brush border" structure and with a membrane similar to the cell membrane lining the rest of the surface facing the lumen.

It must be pointed out that the "brush border" structure is sufficiently complicated to make it extremely difficult to accept a definite opinion regarding the detailed organization. Further studies are urgently required.

The vacuoles and big granules of the intermediate cell zone. Big vacuoles and granules consistently are present in the intermediate cell zone. The vacuoles are not due to improper fixation. They correspond to the granules which are best studied in the fluorescence microscope due to their containing yellow fluorescent substances (Fig. 11). These substances are easily dissolved in water and, therefore, are removed during the fixation and embedding. The granules then are replaced by vacuoles.

These vacuoles appear in the EMG's as almost empty, about 0.5 μ wide spaces lined by a dense margin. This margin exhibits a sharp, distinct contour against the surrounding cytoplasm. Towards the interior of the vacuoles there is a gradual decrease of the opacity. This fact might be due to some of the material forming the original granules not being dissolved but remaining as a thin coating of the vacuoles or there may exist an organized surface structure in the original granules.

Often there remains some material forming an inner granulum attached to the wall of the vacuole. In addition to these vacuoles there exist big granules in the intermediate zone and between the apical ends of the mitochondria (Figs. 7 and 8). The largest granules measure 0.5–0.7 μ in diameter. These granules may easily be distinguished from cross-cut mitochondria due to their quite different structure. They are surrounded by a

TABLE IV

The dimensions of the nuclear double membranes.
Each figure in the second and third column are mean values of 24 measurements.

Nucleus number	Total thickness Å	Distance between centers of the constituent single membranes Å	Calculated thickness of the single membranes Å	Calculated height of the space between the single membranes Å
1	236	164	72	92
2	251	192	59	133
3	224	167	57	110
4	210	142	68	74
5	236	166	70	96
6	220	162	58	104
7	218	152	66	86
8	226	161	65	96
9	222	160	62	98
10	239	173	66	107
Mean	230 ± 4[1]	165 ± 4[2]	65	100

well marked membrane which at the highest resolution obtained in this study may appear as very thin double membranes. The total thickness of these double membranes varies between 50 and 90 Å.

In contrast to the mitochondria the material of the granules exhibit an obvious grainy appearance. Each granule also contains a larger or smaller, extremely dense inner granule, which in contrast to the dense spots of the mitochondria show a sharply defined boundary (Fig. 8). The contrast of these inner granules is pronounced due to an extensive reduction of osmium tetroxide.

The nuclei. The nuclear membrane is a double membrane, the constituent dense single membranes being separated by a less dense zone.

The total thickness of the nuclear membrane is 230 ± 4 Å. The distance between the centers of the two single membranes is 160 ± 4 Å. Each mean value is based on 240 measurements on 10 nuclear membranes. The calculated thickness of the single membranes will be 60–70 Å and the height of the space in between the two single membranes is about 100 Å (Table IV).

The chromatin is concentrated towards the nuclear membrane and some indications of a granular structure of the chromatin with a somewhat uniform grain size seem to justify further analysis.

[1] The coefficient of variation 12 per cent.
[2] The coefficient of variation 13 per cent.

DISCUSSION

Several new facts regarding the structure of the tubular cells of the kidney have been revealed through the present electron microscopic analysis.

The cytoplasm of these cells is highly organized with a series of different well-defined structures, which reduce the amount of what can be classified as the ground substance of the cytoplasm to a rather restricted proportion. In order to facilitate the description of topographic relationships within the cells the proximal tubular cells have been divided in three main parts: *The basal zone*, characterized by the densely packed mitochondria and reaching from the basement membrane to the apical ends of the mitochondria. This zone contains the nucleus. *The apical zone*, corresponding to the zone of the "brush border" and *the intermediate zone*, constituting a zone of varying height in different sections of the proximal convoluted tubule and characterized through the existence of big granules, vacuoles and the basal rod-shaped extensions of the "brush border" components.

The intracellular cell membranes. A striking feature is the partial separation of the cytoplasm in compartments through intracellular membranes with close relations to the mitochondria. A similar arrangement has also been observed in the excretory cells of the mouse pancreas as a very highly developed system of closely packed double membranes (22).

It is quite obvious that we are dealing with membranes and not filaments from the fact that they may be followed without interruptions through the whole basal cell zone, the chance to hit a filament so exactly along its entire length being negligible. In addition there have never been any indications of cross-cut filaments.

The intracellular membranes reach the surface of the cytoplasm facing the basement membrane but the greater part of them is embedded in the ground substance of the cytoplasm. They, therefore, should be distinguished from the outer plasma membranes lining each cell and which may consistently be observed in well preserved specimens. These outer plasma membranes extend from the basement membrane the whole distance to the "brush border" in a quite regular manner.

It seems obvious that these intracellular membranes correspond to the "interdigitating cell membranes" of Pease and Baker (15) and Dalton (2) and the intracellular filaments or lamellae of Dalton (2). The micrographs of these authors are, however, not of the quality to allow a detailed description and a correct interpretation of these structures.

The mitochondria. The basal striations of the proximal and distal con-

voluted tubular cells exhibit all the characteristics of mitochondria: they constitute small cytoplasmic corpuscles (according to a light microscopic scale), which stain supravitally, with Janus Green B (according to an unpublished investigation of L. Elfvin in our laboratory) and stain bluish black with Heidenhains iron-hematoxylin stain (Fig. 10). There has been a discussion regarding their form and functional conditions. These problems have been extensively dealt with in the monograph of Sjöstrand (18).

When investigating well-preserved material, for example frozen-dried or osmium fixed kidney tissue, the mitochondria appear as long filaments (Fig. 10).

A granular appearance of the mitochondria may be ascribed to damage, which can easily be caused by the intravenous injections of sodium chloride, hypertonic dextrose, diodrast or may very easily result from postmortal changes. Perfusion of the kidney with saline before fixation represents one of the most drastic methods to produce artefacts. This treatment also prolongs the time between the interruption of the blood supply to the kidney and the action of the fixing agent and, therefore, increases the time for postmortal disorganization to take place.

When examined at high power magnification with the light microscope, the specimens fixed with osmium tetroxide according to the method used in this investigation are identical to the best preserved frozen-dried specimens. We, therefore, should expect consistently to find long filamentous mitochondria. The mitochondria appear, however, in the EMG's as particles of varying length. Partially this must be due to an irregular wavy course of the mitochondria but it may also depend upon the existence of shorter mitochondria arranged in rows within the intracellular compartments.

The investigation has demonstrated an internal structure within the mitochondria, which as far as we know has not been described before. The system of transversally oriented double membranes and the clear cut demonstration of a similar outer membrane around the mitochondria indicate a high degree of organization of these cell organelles. The precise measuring of the thickness of these membranes and of their spacing make it possible to investigate structural changes within the mitochondria on a quantitative basis.

A similar structure of the mitochondria has been observed in the guinea pig retinal rods, in mouse pancreas and in the surface epithelium of the mouse intestines (21).

A rather high concentration of bound riboflavin in the basal cell zone, of the proximal convoluted tubule has been demonstrated through fluorescence

microspectrography (23). The riboflavin seems to be localized to the mitochondria and presumably reflects the localization of yellow enzymes. The structure of the mitochondria make an oriented and organized distribution of the enzyme molecules within the mitochondria possible.

The "brush border." This name refers to the appearance of the apical cell zone of the proximal convoluted tubular cells when distorted through improper fixation. When properly fixed with osmium tetroxide or freeze-drying (18) this cell zone is optically homogeneous when examined in the light microscope in light field (Fig. 10) as well as in dark field (23). Only in the most distal part of the proximal convoluted tubule is there any evidence of a coarser structure, which appears like a seam with numerous pores.

The homogeneous apical cell zone exhibits form birefringeance which is positive with respect to a direction perpendicular to the cell surface and with the optical axis in the same direction indicating anisodiametric particles oriented perpendicularly to the cell surface (18). The electron microscope observations are in full agreement with these observations. The fine and tightly packed ducts of this cell zone cannot be resolved with the light microscope. These ducts are closed all around and the walls of each duct continues at the base with a compact curved rod.

The arrangement revealed does not seem to favour the idea that the functional significance of this structure would be to increase the effective surface of the cells towards the tubular lumen. Observations made by S. Helander (11) on the excretion of sulfanilamide derivatives through the kidney indicate that these substances are absorbed to and concentrated within this cell zone. The ultrastructure seems to be in good harmony with the idea that this cell zone is adapted to absorb components from the filtrate.

The structure of the "brush border" as described here does not agree with the descriptions of Pease and Baker (15) and Dalton (2) but the discrepancies are easily explained by the differences regarding the preservation of the tissue and regarding the resolution of the micrographs.

The granules and vacuoles of the intermediate cell zone. Granules and vacuoles have been observed in the intermediate cell zone and have been described in various manners (see (18)). Sjöstrand found that these structures are derived from yellow fluorescent granules (Fig. 11) of different composition in different parts of the proximal convoluted tubule. These granules contain water soluble fluorescent components and most of them appear as vacuoles in sections stained according to ordinary histologic techniques (Fig. 10). Some of them exhibit a different chemical composition and

reduce osmium tetroxide appearing as dark granules after osmium fixation. These granules, more or less distorted, seem to constitute the Golgi apparatus of these cells (J. Rhodin, unpublished observations).

The electron microscopic observations reported here demonstrate the existence of empty vacuoles as well as granules exhibiting more or less intense contrast due to the reduction of osmium tetroxide. These vacuoles and granules correspond to the yellow fluorescent granules. According to the fluorescence microscopic analysis (18) their amount varies with the functional conditions which points to their functional significance.

The nuclear membrane. Little evidence of an organized structure within the nucleus have occurred. This might depend upon the fixation technique not being well enough adapted for the conditions characteristic of the nucleus.

The nuclear membrane appears as a double membrane of slightly different dimensions than the intracellular cytoplasmic membranes. Observations indicating a double nuclear membrane have been reported by Callan and Tomlin (1) and by Hartmann (10).

The basement membrane. There is a zone of condensed cytoplasm lining the basal surface of the tubular cells and an about 200 Å wide space separating this surface structure from the basement membrane. In addition the intracellular membranes assist in separating the cytoplasm from the basement membrane. The interstitial side of the basement membrane is always covered by endothelial cells of the blood capillaries or by connective tissue. The basement membrane, therefore, appears as a rather independent structure and it seems difficult from these facts to conclude whether it represents an integral part of the tubular cells or of the connective tissue.

The structure of the double membranes. Double membranes are frequently occurring structures of the cells. In the tubular cells they have been observed as constituting cell membranes, nuclear membranes, intracellular membranes, outer and inner mitochondria membranes and as membranes lining cytoplasmic granules. They have also been observed in the excretory pancreas cells forming the corresponding structures (22). In the latter cells intracellular double membranes have been observed in osmium fixed as well as in frozen-dried preserved material. The characteristic appearance of these membranes as double membranes, therefore, seems not to be restricted to osmium fixed material. On the other hand it does seem obvious that the osmium fixation enhances the contrast of the membranes.

What does this appearance of the membranes mean? The resolution of the micrographs on which the present description is based is sufficiently high to reveal that the two constituent membranes forming a double mem-

brane are well defined structures with sufficient deviations from a parallel course to demonstrate a certain mutual independence (Fig. 2 b). They are, on the other hand, parallel enough to justify considering the two membranes with the space in between as representing a structural unit. The micrographs are close to focus pictures from through-focus series. This constitutes an additional fact that excludes that we are dealing with a contour phenomenon due to a diffraction effect.

Another possibility would be that we are dealing with single membranes which appear as double due to an oblique orientation with respect to the direction of the beam with an accentuation of the contrast at the cut margins in the upper and lower surfaces of the sections. This possibility is excluded by several facts: The well defined lines are separated by a zone of too low density to correspond to the density of an obliquely oriented membrane. The characteristic spacings measured are rather constant for a certain kind of membrane but varies with the localization of the membranes whether in mitochondria or in cytoplasm and is independent of variations in the thickness of the sections. Comparable membranes exist which do not exhibit any double character. It, therefore, seems justified to assume that the double membranes are composed of two opaque layers or thin membranes separated by a less opaque zone.

The experience from studying the structure of the outer segments of the retinal rods (19, 24, 25, 26) has revealed that protein membranes when cut across exhibit a marked contrast especially after osmium fixation. The osmium appears as an efficient electron stain for such membranes.

The following interpretation of the structure of the double membranes may, therefore, easily be offered. The two constituent single membranes of the double membranes are mainly of protein nature and the zone in between corresponds to a layer of lipids. The density of the protein membranes is enhanced due to the reduction of osmium tetroxide and the density of the lipid layer is lowered due to a partial dissolution of the lipids during the embedding. This interpretation is supported by the measured spacings of the different membranes. The thickness of the presumed protein membranes is 45 Å for the mitochondria membranes, 60 Å for the nuclear and the cell membranes and 80 Å for the intracellular membranes. The thickness for the presumed lipid layers is 70 Å for the mitochondria membranes, 100 Å for the nuclear membranes and 110 Å for the intracellular membranes. The figure for the cell membranes 160 Å is based on too few measurements to be significant.

The structure of the plasma membrane has been studied with indirect

methods and is discussed by Danielli (5) in a recent review. Those indirect findings are in harmony with the assumption that the basic structure of the plasma membrane would be a continuous lipid layer to which structure layers of adsorbed protein are added on both sides to account for the low interfacial pressure found and for the stability of the system.

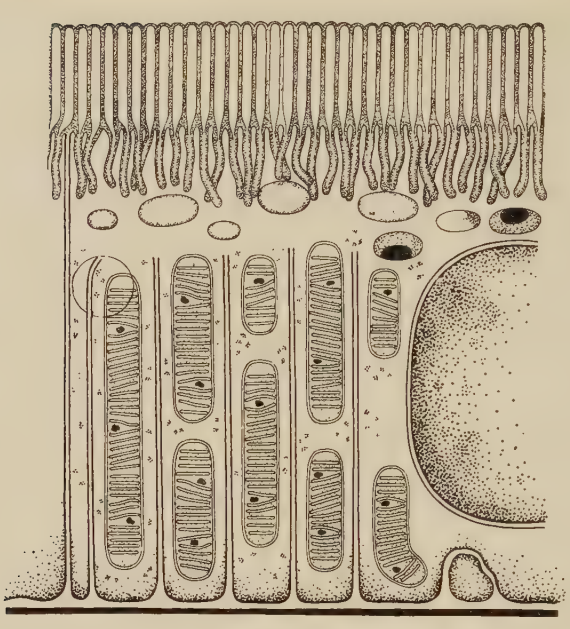
This theory is in good agreement with the observations made in this study regarding different intracellular membranes provided that the interpretation presented here is correct. The indirect estimations of the proposed lipid layer of the plasma membrane (6, 7, 9, 16, 17) indicate that the thickness of the lipid layer would correspond to 2–4 molecules and Danielli concludes that there is a reasonable upper limit of the thickness of this layer of about 100 Å.

This assumed thickness of the lipid layer of the plasma membrane is in good agreement with the measured height of the space between the dense membranes of the double membranes of different location in the tubular cells.

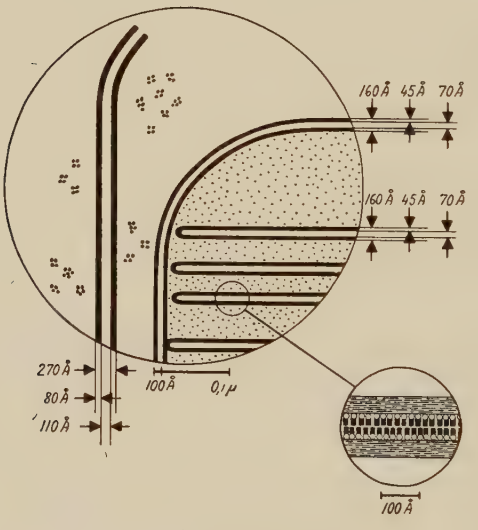
The proposed interpretation of the observations described here has to be tested by further experimental work and the measurements must also be extended to different experimental conditions to analyze how the dimensions are influenced by shrinkage or swelling during fixation and embedding. Measurements that vary as much as they do for the intracellular membranes must be considered rather uncertain. The demonstration of freeze-drying as a useful technique for the preservation of comparable membranes in the pancreas cells (22) is of a certain value in this connection.

The ground substance of the cytoplasm. The chance of preserving the structure by fixation seems to increase with the degree of organization. In highly organized structures as mitochondria the total amount of forces in action to keep the molecules arranged, in for example membranes, may be assumed to be considerable because many molecules are interacting in stabilizing the same structure. In such cases the fixing agent may represent an insufficient force to tear and break the structural relations. But dealing with the ground substance of the cytoplasm the structure, if present, is so delicate, the binding forces so comparatively few between adjacent molecules that the preservation is much more hazardous. It, therefore, seems unsafe to discuss the organization of the ground substance of the cytoplasm without the greatest reservation.

In the ground substance particles with a diameter of about 40 Å, that is of molecular dimensions, have been observed in small groups, each group measuring about 100 Å in diameter. The constancy of this structure and the regular distribution might well indicate a fundamental organization of the ground substance or might reflect, for instance, the way in which protein material is precipitated by osmium tetroxide.



Textfig. 2.



Textfig. 3.

Textfigs. 2 and 3. Schematic presentation of the observations made on proximal convoluted tubule cells by means of electron microscopy. Textfig. 3 represents an enlargement of the area encircled in Textfig. 2 (at the arrow at the left side of the drawing).

The same cautious evaluation should be applied to the irregularly oriented thin fibrils which sometimes form an irregular network in between the mitochondria.

Textfigs. 2 and 3 represent an attempt to summarize in a diagram the observations described in this paper.

SUMMARY

The ultrastructure of the cells of the proximal convoluted tubules of the mouse kidney has been studied by means of electron microscopy at a fairly high resolution (35 Å) on ultra-thin sections. The tissue was fixed in buffered (pH 7.2) osmium tetroxide according to Pallade and embedded in n-butyl-methacrylate.

1. Towards the basement membrane the cell membrane appears as a thin zone of condensed cytoplasm. An about 200 Å wide space separates this cytoplasmic zone from the basement membrane. Between adjacent cells the separating membrane consists of an about 160 Å thick double membrane, which connects to the "brush border" structure.

2. The cytoplasm of the basal cell zone is divided into compartments by means of double membranes here called intracellular cytoplasmic membranes. They surround the mitochondria and at the basal cell surface they assist in lining the cytoplasm towards the basement membrane. The mean thickness of these membranes is 270 Å, the thickness of each constituent single membrane is 80 Å and the height of the space in between the two membranes is 110 Å.

3. The mitochondria are rod-shaped. They are lined by a 160 Å thick double membrane consisting of two about 45 Å thick membranes separated by a 70 Å wide space. In the interior of the mitochondria there are transversally oriented double membranes organized in a similar way as the outer mitochondria membrane and mutually separated by 170–470 Å wide spaces. Between these internal membranes areas exhibiting a pronounced contrast due to a heavy reduction of osmium tetroxide may be found.

4. The "brush border" consists of densely arranged cylindrical "ducts." The "ducts" are 600 Å wide and 1.0–1.5 μ long and lined by a thin dense wall. These ducts are closed towards the tubular lumen. They are also closed at their base where the walls continue in curved rods. The "ducts" are oriented perpendicularly to the cell surface facing the lumen and in cross sections they closely resemble the structure of a honey-comb with the exception that the tubes approach a circular, more than a hexagonal cross section.

5. In the intermediate cell zone there are granules and vacuoles, corresponding to the previously described fluorescent granules (18). These structures exhibit a well marked membrane surrounding the vacuole or the granule. The granules contain inner granules which are extremely dense due to heavy reduction of osmium tetroxide. The granules of this cell zone correspond to the osmium tetroxide reducing granules observed in the light microscope and which have been classified as constituting the Golgi apparatus of these cells.

6. The nuclei are lined by a 230 Å double membrane, the constituent single membranes measuring 60 Å in thickness and the height of the space in between these single membranes 100 Å.

7. The structure of the characteristic double membranes observed as cell membranes, intracellular cytoplasmic membranes, outer and inner mitochondria membranes and nuclear membranes is discussed and it is tentatively assumed that they are composed of two protein membranes separated by multilayers of lipid molecules.

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INVERSE RELATIONSHIP BETWEEN CHROMOSOME PLOIDY AND HOST-SPECIFICITY OF SIXTEEN TRANSPLANTABLE TUMORS

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The conversion of a representative variety of solid mouse neoplasms into ascites, recently accomplished by Klein (14), Goldie and Felix (9), and others, has made available "nearly pure cultures" (25) of malignant cells freely suspended in peritoneal exudate. This material includes several tumors derived from inbred strains and has, therefore, obvious advantages over the older Ehrlich mouse carcinoma and Yoshida rat sarcoma for immunogenetic work. Histocompatibility studies may be coupled with simultaneous cytological analysis, since ascites growths have proved particularly amenable to observations of mitotic details (18) and to exact chromosome counting.

Excellent agreement has been obtained between modal ploidy, as determined through chromosome counts in various near-diploid and tetraploid tumors (13, 17) and average values of DNA per cell or per isolated nucleus found by the DNA phosphorus method (15) and by ultraviolet spectrophotometry (8, 16). The absence of confusing necrotic and host-stromal elements, such as occur in most solid cancers, makes the chromosome constitution (ploidy valence) of ascites cells a clear-cut criterion which may be doubly ascertained by cytological and chemical techniques and can be reliably correlated with certain functional characteristics. The present report deals with the interrelationship of ploidy (2n versus 4n), antigenicity, and host-range, previously observed for seven mouse ascites (11) and now extended to a larger series.

MATERIALS AND PROCEDURES

The tumors investigated by us, including recently established ascites as well as neoplasms with a long transplantation record, have been described by Klein and others (1, 2, 7, 9, 10, 15, 18, 22, 23, 24, 26). They were selected on the basis of the following properties: (a) representative variety of histological types, comprising four lymphomas, five carcinomas and six sarcomas; (b) progressive growth in at

least one host-genotype resulting in 100 per cent mortality; (c) relatively rapid growth rate; (d) serial transplantability in the ascites form; (e) low percentage of non-malignant mesothelial cells, histiocytes, polymorphonuclear leukocytes and lymphocytes in differential counts of the cell population (15).

The DBA lymphoma, 6C3HED lymphosarcoma, TA3 mammary adenocarcinoma, MC1M rhabdomyosarcoma, Krebs 2 anaplastic carcinoma, Ehrlich carcinoma, and sarcoma 37 were carried at this laboratory for 66, 68, 64, 30, 92, 86 and 86 intraperitoneal transfer generations respectively, and supplied the bulk of our data (Table II). Supporting evidence from nine further tumors is summarized in Table III which combines relevant information from the literature, unpublished findings of several investigators and chromosome counts or compatibility tests performed here (indicated by asterisks).

The genetically indifferent neoplasms were propagated in A/He, C3H/St and Swiss mice; the strictly or partly host-specific ascites were maintained in the inbred strains of origin and, where possible, also as sublines in one or two susceptible foreign stocks.

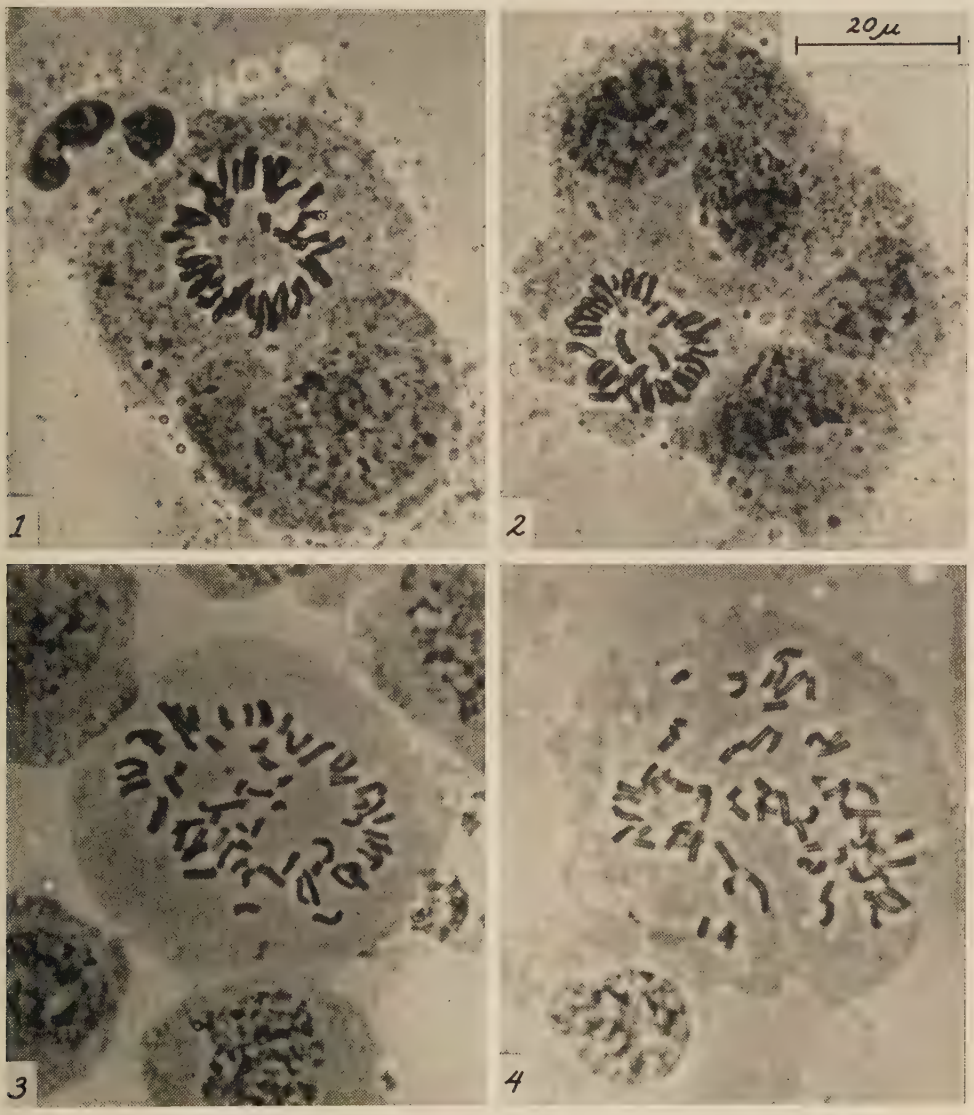
Host-range was determined by challenge with multiples of the normal 100 per cent lethal dosage of freshly tapped undiluted ascites fluid, with the current passages in the home-strains serving as controls. In these tests, groups of from 10 to over 100 male and female mice 2–4 months old of the A/He, A/St, $C3H_f$/He, C3H/St, C57BL, C58/Md, DBA/1, DBA/2, 129/Rr inbred lines and of the Swiss and other heterogeneous stocks were inoculated with tumor and were kept for three months thereafter to observe takes, regressions, and mortality. Serial passage of the Yoshida rat sarcoma was attempted in 200 gram Wistar and Lankenau rats of both sexes.

Four of the mouse tumors which had arisen in inbred genotypes (Table I) were subjected to genetic histocompatibility tests by inoculation into appropriate F_2 and back-cross hybrids between the susceptible stock of origin and totally resistant lines. The number of *H*-factors governing the antigenic relationship between graft and host were calculated from the survival percentages.

For the cytological analysis, droplets of ascites suspension were fixed and stained on slides in aceto-orcein and pressed out with bibulous paper between the siliconed surfaces of slide and coverslip (unpublished method of J. Schultz). All chromosome counts for the ploidy surveys were made on well-spread metaphase plates under immersion with or without phase contrast and with frequent checks by camera lucida drawings. The counts are either exact or in rare cases subject to an error not exceeding ± 2 chromosomes for the diploid tumors and ± 1 for the larger-celled tetraploid ascites (17).

RESULTS

The distribution of chromosome numbers, based on representative samples of 50–100 critically countable metaphases (Figs. 1–4), showed each tumor to be characterized by a distinct ploidy pattern. The modal values were either diploid, which is 40 in the mouse, or centered around the tetraploid region. The chromosomal constitutions of the various diploid and tetraploid ascites are typified by the histograms for DBA lymphoma, 6C3HED lymphosarcoma, Ehrlich carcinoma and Krebs 2 carcinoma which have been anal-



Figs. 1 and 2. Diploid metaphases of the TA3 mammary adenocarcinoma ascites which tends to form small cell clusters suspended in peritoneal exudate.
Fig. 3. Aneuploid metaphase of the Krebs 2 anaplastic carcinoma ascites containing 67 chromosomes. Like most other ascites, the Krebs 2 grows as a free cell suspension.
Fig. 4. Tetraploid Krebs 2 cell. All photomicrographs are of aceto-orcein smears, taken with a dark-phase contrast immersion objective.

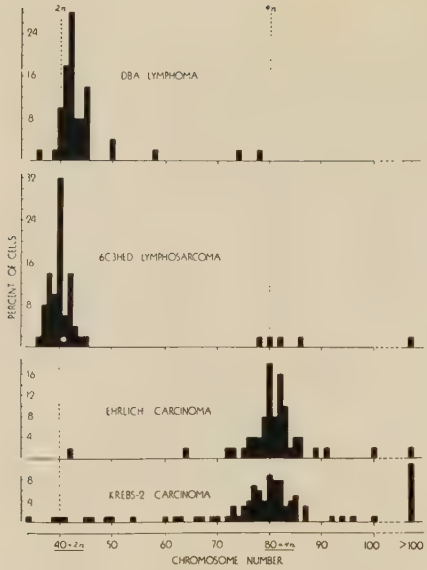


Fig. 5. Histograms of the chromosome numbers in four mouse ascites tumors. 40=2n; 80=4n. All cells with chromosome numbers exceeding 100 are combined in one column. This analysis is based on 250 critically countable metaphase plates (50 each in DBA, 6C3HED, and Ehrlich; 100 in Krebs 2).

yzed most extensively (Fig. 5). These data are in complete accord with the corresponding single or double values arrived at by chemical or spectrophotometric assay of DNA/cell (7, 15, 16, 24 and Shelton, unpubl.).

The observed variation in chromosome number was found to be influenced by minor anaphase disturbances, such as non-disjunction or lagging, chromosome doubling by c-mitotic or endo-mitotic mechanisms, and the formation of multipolar spindles. In spite of this mitotic imbalance, each tumor has retained its individual ploidy pattern through many successive transplant generations, as shown by repeated intermittent sampling. Under continuous natural selection, cells with the more balanced sets of chromosomes in the regions of 2n, 4n and even 8n (17) appeared to have competitive advantages over the aberrant intermediate types.

Four tumours which had arisen in inbred strains were tested for segregation of antigenic loci in a total of 1 042 F_2 and resistant back-cross mice (Table I). It is interesting that the three highly host-specific diploid ascites required 2 (in one case possibly 1) histocompatibility genes, whereas the sub-tetraploid MC1M, with a capacity for growth in some foreign strains but not in others, gave a one-factor ratio. Tumors like the Ehrlich, Krebs 2, S37 and S180 which are genetically indifferent, seem to lack antigens of sufficient potency to evoke a protective antibody titer in the many host-genotypes which regularly succumb to them. This does not imply that agglutino-

TABLE I

Histocompatibility genes determining the antigenicity of four mouse ascites tumors.

Tumor	Susceptible × resistant parent stocks	Resistant BC test				F_2 test				H-Factors Required
		Number of Mice	Tumor Deaths	Per cent Takes	Per cent Exp.	Number of Mice	Tumor Deaths	Per cent Takes	Per cent Exp.	
DBA lymphoma	DBA/2 × C3H$_f$/He	428	110	25.7	25.0	207	113	54.6	56.2	2
TA3 adenocarc. . .	A/He × C3H/St	100	28	28.0	25.0	100	65	65.0	56.2	2
6C3HED lymphosarc. . . .	C3H/St × A/He	–	–	–	–	100	64	64.0	56.2	1 or 2
MC1M rhabdomyosarc.	C3H$_f$/He × DBA/2	–	–	–	–	107	76	71.0	75.0	1

TABLE II

Relationship between chromosome constitution, histocompatibility genes, and host-specificity in a representative series of diploid and tetraploid ascites tumors.

Mouse ascites tumor	Modal ploidy	H-factors required	Origin	Host-strains in which tumor grows progressively	Per cent tumor deaths in foreign strains
DBA lymphoma . . .	2n	2	spont. in DBA/2 ♀	subline DBA/2	0
6C3HED lymphosarcoma	2n	1 or 2	induced in C3H/St ♀	C3H/St, C3H$_f$/He	0
TA3 mammary adenocarcinoma	2n	2	spont. in A/He ♀	A/He, A/St	0
MC1M rhabdomyosarcoma	sub-4n	1	induced in C3H ♂	C3H$_f$/He, A/St, A/He, Swiss	53, 64
Krebs 2 carcinoma	4n	0	spont. in non-inbred stock	A/He, A/St, C57BL, C58/Md, C3H$_f$/He, C3H/St, DBA/2, 129/Rr, Swiss	100
Ehrlich carcinoma	4n	0			100
Sarcoma 37	4n	0			100

gens are absent or that these growths are non-antigenic with respect to *all* host-strains. The I-strain, for instance, is relatively resistant to S37 (1), and regressions of S180 are frequent in Rockland and some other mice.

The host-range experiments which furnished the information in column 5 of Tables II and III will be outlined for only two examples, 6C3HED lympho-

TABLE III

Influence of ploidy on host-specificity in nine further tumors with known chromosome constitution.

Tumor	Modal ploidy	Method of ploidy determination	Origin	Host-strains showing progressive tumor growth	References
Yoshida sarcoma (ascites)	2n	chromosome counts and DNAP/cell	induced in ♂ Japanese "stock" rat	only rat strains derived from stock or origin[1]	Yoshida (26), Klein (15), Makino and Kano (20), Kinosita (unpubl.)
A-lymphoma No. 2 (solid and ascites)	2n	DNAP/cell and chromosome counts[1]	induced in ♀ A/He mouse	A-strain only[1]	Shelton (22, and unpubl.)
A-lymphoma No. 1 (solid and ascites)	4n	DNAP/cell and chromosome counts[1]	induced in ♀ A/He mouse	A/He, A/St., A/Jax, DBA/2 (85 per cent)[1]	Shelton (22, and unpubl.) Carnes *et al.* (7)
Sarcoma DBA49 (solid and ascites)	ca 40 per cent 4n	chromosome counts[1]	spont. in ♀ DBA mouse	DBA, BALB/c (90 per cent)	Barret (2)
Carcinoma 15091a (solid and ascites)	4n	DNAP/cell	spont. in ♀ A/St mouse	A, DBA (31 per cent), also RI, C57BL, C57BR, BALB/c, C3H	Klein (15) Snell (23) Barrett (2)
HS1 sarcoma (ascites)	4n	chromosome counts[1]	spont. in ♀ Swiss mouse	Swiss, C3Hf/He, C3H/St, A/He, C57BL	Williams (unpubl.) Barrett (unpubl.)
Sarcoma 180 (solid and ascites)	4n	microphotometry of Feulgen nucleal reaction	spont. in Crocker mouse	most strains of mice (60–100 per cent)[1]	Carnes *et al.* (7)
Hall carcinoma (solid)	40 per cent 4n and higher; 60 per cent 2n	microphotometry of Feulgen nucleal reaction	spont. in ♀ Swiss mouse	various mouse stocks; heterologous growth in rats often lethal	Hall (10) Swift (24)
S91 melanoma (solid)	4n	microphotometry of Feulgen nucleal reaction	spont. in ♀ DBA mouse	DBA, BALB/c (80–100 per cent)	Swift (24) Loustalot *et al.* (19)

[1] Data obtained or verified at this laboratory.

sarcoma and MC1M rhabdomyosarcoma. While some of the diploid neoplasms, notably the DBA lymphoma and the TA3 carcinoma, did not even give temporary takes in six incompatible genotypes, the equally specific diploid 6C3HED (C3H origin) was successfully subcultured through now 18 serial passages in the unrelated Swiss stock. Throughout these transfers the ascites "took" as a blood-free cell suspension and without signs of solid tumor formation or infiltration in 112 Swiss mice and later regressed without a single recurrence or death. Morphology, chromosome constitution, and mitotic index of the 6C3HED ascites during 18 generations in the genetically strange environment remained unchanged as compared with smears from uninterrupted passage in the home-strain. Timing was crucial in the propagation of the Swiss subline of 6C3HED; it was necessary to transplant every 7–10 days, as resorption in the current passage was complete soon thereafter.

In contrast with these results, the subtetraploid MC1M sarcoma—also of C3H origin and unable to grow in the C58/Md, DBA/2 and 129/Rr strains—killed 67 out of 104 Swiss hosts in 21 serial passages and 34 of 64 A/He and A/St mice in 16 serial transfers. Mean survival time of the animals which died with tumors was 22.6 days in $C3H_f$/He, 24.7 in F_2 hybrids (Table I), 32.8 in Swiss, and 44.0 in A/He or A/St.

A group of 107 [susceptible $C3H_f$/He×resistant DBA/2] F_2 hybrids (Table I), all inoculated at the same time with MC1M, had shown 71 per cent takes followed by death; this indicated a single *H*-factor difference between the C3H-derived rhabdomyosarcoma cells and the non-susceptible DBA/2 strain. In the Swiss and A/He sublines of MC1M, tumor mortality for the first five serial transfers was only 22.2 per cent and 25.0 per cent respectively; but during passages 6–10 in Swiss and 6–11 in A/He a rapid shift (immunologic selection) occurred. The incidence of progressive takes rose abruptly to 73.3 per cent in Swiss and 76.2 per cent in A/He and remained at that level (approximating a dominant F_2 one-factor ratio) during subsequent generations in both foreign stocks. None of the diploid tumors tested exhibited a similar capacity for adjustment across genotypic barriers.

When the extensive information gained from the host-range experiments is considered side by side with what we had learned about chromosome numbers and gene requirements (Tables II and III), a surprisingly consistent relationship between ploidy and strain-specificity emerges from the data: The neoplasms with diploid chromosome modality were lethal only for their particular strains of origin or for F_1, F_2 and BC hybrids carrying the necessary histocompatibility genes inherited from the susceptible parent stock. Diploid tumors always regressed in unrelated host-genotypes. Con-

versely, all the cancers in the near-tetraploid category lacked rigid specificity. Their growth-limits extended beyond the genotypes in which they had arisen into at least one but usually several foreign strains, or they were entirely indifferent with regard to transplantability.

DISCUSSION

The combined immunogenetic and cytological approach to problems of specificity in a "spectrum" of ascites tumors permits a generalization (11) concerning the histocompatibility relationship between graft and host (12):

Tumors with diploid chromosome modality are completely strain-specific. Tumors with near-tetraploid constitution exhibit varying degrees of genetic indifference with regard to their host-requirements.

A systematic search has so far netted no exception which oversteps the apparently rigid line of demarcation between the diploid and the tetraploid categories. The wider transplantability of the latter in our representative series is independent of growth rate, virulence, tissue of origin, and parent strain. Rapidly growing invasive neoplasms occur both in the 2n and 4n class. Both ploidy types include lymphomas, carcinomas, and sarcomas; spontaneous and induced growths; and the inbred strains of origin are overlapping in some instances. Thus, 6C3HED (2n) and MC1M (sub-4n) were chemically induced in C3H mice (15). Lymphomas No. 2 (2n) and No. 1 (4n) both arose in July 1946 in two six-months old A/He females which had received the same dosage of total body x-radiation at birth (22), and are comparable for the purposes of the present correlation.

The argument that the tetraploid Krebs 2, Ehrlich S37, S180, HS1, and the Hall carcinoma originated in hybrid mice, hence are prone to be genetically non-specific, is invalidated by what is known about transplantation of F_1 tissue. Survival of a graft from a hybrid donor usually depends on histocompatibility genes contributed by both parental stocks and one should expect such heterozygous implants to contain more (not fewer) antigenic factors than the pure-line diploid tumors. Furthermore, the diploid Yoshida sarcoma (Table III) is quite restricted in its strain-compatibility, despite its derivation from a non-inbred source.

As for duration of serial passage, this also has no important bearing on decline in host-specificity (except that *H*-mutants may accumulate with time). While the Ehrlich (4n) and S37 (4n) are relatively ancient, the non-specific MC1M (sub-4n) is three years younger than the specific 6C3HED (2n) and Lymphoma No. 1 (4n) and No. 2 (2n) are of identical age.

Nevertheless, the histories of the fifteen tumors examined here have been sufficiently varied to call for more critical proof of the constancy of the inverse relationship between ploidy and histocompatibility.

The influence of antigenic overlapping between certain inbred strains (23) on the observed host-range must be taken into account. Thus, the progressive growth of sarcoma DBA49 and S91 melanoma (both of DBA origin) in BALB/c mice could be attributed to the presence of the $H\text{-}2^{d}$ allele (one of six antigenic alleles at the *H*-2 locus) in both DBA/2 and BALB/c, rather than to the tetraploid chromosome constitution of DBA49 and S91. Snell (23 and unpublished data) has found that all F_1 hybrid mice of the genotype $H\text{-}2^{d}/H\text{-}2^{k}$ are susceptible to strain A($H\text{-}2^{dk}/H\text{-}2^{dk}$) tumor 15091 a (4n) regardless of which of the *d* and *k* stocks is involved in the cross. Similarly, we have obtained 100 per cent takes with the tetraploid A-strain Lymphoma No. 1 in F_1 hybrids between DBA/2 ($H\text{-}2^{d}$) and C3H($H\text{-}2^{k}$), but also a high percentage of takes in the pure DBA/2 stock. Tests are now under way to determine whether the diploid TA3 carcinoma and Lymphoma No. 2 (both of A-strain origin) will give comparable results.

Additional evidence for the role of ploidy in histocompatibility is being sought through current attempts to establish single-cell clonal sublines of one and the same ascites differing in chromosome constitution and, presumably, in host range.

A simple duplication of the diploid chromosome set is probably not the essential factor in the loss of antigenicity; for if this were so, the rare tetraploid cells in a predominantly diploid ascites (Fig. 5) should occasionally survive and give rise to a new cell population after inoculation into a foreign strain. Ample opportunity for immunological selection of this sort was provided during the prolonged serial passage of 6C3HED (C3H origin) in Swiss mice where regression was 100 per cent. On the other hand, polyploid cells were more frequent in heteroplastic transplants of the Yoshida sarcoma (2n) than in homoplastic transfer (27) which suggests an at least temporary survival value under immunologically hostile conditions.

Bieselc, Poyner and Painter (5) have discussed the possible bearing on malignancy of quantitative changes in the gene balance of cancer cells with multi-stranded chromosomes (4, 5, 6). The contention –based on cytologically unfavorable solid tumors—that "the cause of a general shift in nuclear volumes in cancerous tissues is more likely to be found in polyteny than in polyploidy" (6) is not born out by the clearly polyploid ascites material. Yet, multiple dosages of loci with and without cumulative effects could bring about drastic shifts in genic equilibrium resulting in phenotypic changes,

such as reduced antigenicity, whether the chromosome constitution is polytene, polyploid, or aneuploid. In a polyploid cell more than two loci are available for each gene. Allelic *H*-factors (23) may, therefore, function in dosages other than those to which they are held in a diploid. The result could be that antigenic end-products are indistinguishable. We have here a plausible chromosomal mechanism for what Gorer has called "antigenic simplification" (12).

Both the modally diploid and tetraploid ascites are complex heteroploids or chromosomal mosaics (3); but the range of viable cell types with quantitative and/or qualitative rearrangements of the total genome is much greater in the case of 4n modal value. Thus, the chance for antigenically neutral chromosome combinations is increased and the partial or complete genetic indifference of tetraploid tumors appears to resolve itself into a problem of immuno-selection from among the more or less compatible units in a malignant cell population.

The biological advantages of polyploidization are not limited to lessened immune reactivity in refractory hosts but apply to other unfavorable conditions. Freezing-thawing, for instance, is survived better by some of the 4n tumors than by members of the 2n class (Hauschka, unpublished). A striking parallel, perhaps somewhat remote at the present stage of inquiry, is found among plants. Although newly produced polyploids in higher plants have generally been inferior in various ways to their low-chromosome predecessors, doubling of genomes theoretically endows a polyploid with a greater capacity for variation in its adjustments. This is reflected in the well-known prevalence of polyploids over diploids in the arctics as compared with more temperate regions; and in the superiority of some artificial tetraploids over the corresponding diploids (e.g., red clover), as judged from total harvest (21). The consistently wider histocompatibility range of the tetraploid tumors is perhaps also comparable with increased self-compatibility described for several polyploids among higher plants. In both cases a barrier of incompatibility has been overcome through polyploidization.

SUMMARY

A representative series of four lymphomas, five carcinomas, one melanoma, and six sarcomas (largely in the ascites form) has been surveyed for chromosome number distribution, growth capacity in foreign strains and, where possible, histocompatibility genes. This comparative analysis, supported by relevant data from other laboratories, has demonstrated a consistent in-

fluence of ploidy (2n versus 4n) upon degree of antigenicity. The diploid neoplasms were entirely strain-specific and always regressed in unrelated lines. The tumors characterized by a tetraploid modal value were able to grow in and kill hosts of one or more foreign genotypes, or even showed complete genetic indifference. This inverse relationship between chromosome number and host-requirements appears to be independent of the tumor's histology, virulence, strain of origin, method of induction, and transplantation history. The findings are suggestive of "immunologic selection" from a relatively narrow (mode near 2n) or wide (mode near 4n) range of viable antigenic variants in a malignant cell population.

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FERTILIZATION AND MEMBRANE FORMATION OF SEA URCHIN EGGS ASPIRATED IN CAPILLARIES

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As to the way of propagation of the impulse caused by the attachment of the spermatozoon to the egg surface, Rothschild and Swann (9) hold the view that the cortical changes following fertilization could be due to the diffusion of a substance derived directly or indirectly from the sperm head, and that the diffusion might not be "intracortical" but "intracellular," i.e. passing through the interior cytoplasm. Runnström and Kriszat (13), on the other hand, assumed a cortical propagation of the impulse, and that the impulse is spread by a chain reaction rather than by pure diffusion. They had observed that unfertilized eggs of *Psammechinus miliaris* deprived of their jelly coat would stick to the glass bottom and that the propagation of the impulse caused by the spermatozoon was inhibited in the part of the egg surface attached to the glass. Also the underlying cytoplasm remained in an "unfertilized" state. In some cases a hernia-like protrusion was formed of this part, when the egg was detached from the glass. This fragment could be fertilized and start development.

The report given below resulted from an attempt at varying the method when attacking the problems mentioned above. The results obtained do not elucidate the pathways of propagation of the activation impulse. Nevertheless they may throw some light on the mode of operation of this impulse.

METHODS

The eggs were first deprived of their jelly coats. In the experiments first carried out this was done by subjecting the eggs for 10–15 seconds to acid sea water (pH 3.5). After this treatment the eggs were, however, rather delicate. They stuck easily to the bottom of the dish, and they often burst when drawn into the narrow pipette. Immediately after fertilization and removal of the fertilization membrane the egg surface was found to be more resistant. The mortality of the eggs treated with the acid sea water and fertilized in the capillary was great. In the later experiments the jelly coat was therefore removed by sucking the eggs slowly through a narrow pipette. As a rule these eggs developed normally also when fertilized in the capillary.

Their surface layer showed the same degree of toughness as that of fertilized eggs. Thus the greater fragility of the unfertilized eggs in the preliminary experiments was due to the use of acid sea water.

The eggs were aspirated into a pipette drawn out into a capillary of about 10 cm length and with an inner diameter of about 60 μ. The sucking was performed with the mouth by aid of a rubber tubing. As a rule three eggs were drawn into the pipette which was moved with the free hand. The pipette was dipped into a sperm solution, and the outer (last) egg brought down to the mouth of the pipette where the outer end of the egg became fertilized. The eggs were sucked up again into a higher position in the pipette and then blown into a drop of sea water under a cover slip. It was controlled that the two eggs inside the outer egg had remained unfertilized, and that they raised the membrane after being fertilized by the spermatozoa which had been moving freely in the capillary between the mouth and the outermost egg.

RESULTS

In 44 out of 51 recorded cases, a fertilization membrane appeared only at the one end of the elongated egg (Fig. 1 a–d). In seven other cases a membrane was elevated at both ends of the egg (Fig. 1 e). Closer examination of 21 cases of the former category gave the result that the membrane appeared at the pole opposite the point of sperm attachment. The membrane is thus formed at the end which preceded when the egg was aspirated into the capillary. Below, this will be designated as the front (anterior or inner) end of the egg. The end of sperm attachment is the hind (posterior or outer) end of the egg.

No membrane was formed from the part of egg surface, which was tightly pressed against the wall of the capillary. The surface did not really stick to the glass, as the egg could be easily moved up and down the capillary by sucking or blowing. There was no sign of injury of the egg surface in contact with the wall of the capillary.

To sum up: Usually no membrane forms at the hind end of the egg, although the spermatozoon is attached at this end. Nor is there any membrane elevation from the part of the egg surface which is in contact with the glass wall. The impulse initiated at the site of sperm entrance propagates to the opposite end of the elongated egg. A fertilization membrane appears thus in the region of the egg which is most distant from the site of initiation of the impulse.

Under normal conditions a contraction occurs at varying extent, particularly around the site of sperm entrance, for references see (12). The same phenomenon occurred also in the present experiments, but no contraction was here observed at the hind end of the egg, where the spermatozoon pen-

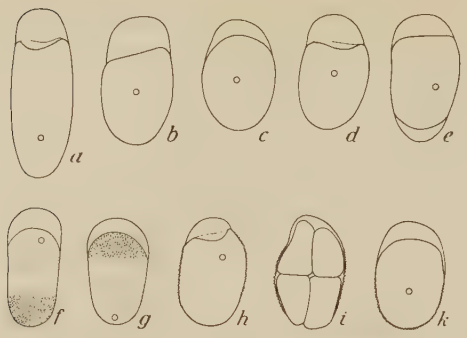

Fig. 1. Formation of fertilization membrane on eggs stretched in a capillary:
a–d. Only at the front end. — e. At both ends.
f, g. In eggs with the cytoplasm stratified by centrifugal force, the membrane is also formed at the front end, irrespective of whether this represents the light (*f*) or the heavy (*g*) pole.
h–k. Illustrating the extension of smooth and granular membrane in two eggs.
h. Below the smooth elevated membrane of the front end, the surface layer is of the typical smooth white type. Along the sides an adhering granulated membrane under which the cortex is spongy white in darkfield. The hind end is smooth and white, without membrane.
i. The same egg in 4-cell stage.
k. The granular layer extends also round the hind end.

etrates. In Fig. 1 b and d it is clearly visible how the front end has assumed a concave instead of a convex shape. This corresponds to the contraction referred to. A membrane elevated uniformly around eggs which were blown out and fertilized immediately upon release from the capillary.

In one experiment eggs were fertilized a few seconds before being sucked up into the capillary. The spermatozoon may have penetrated at any point in relation to the axis of elongation. Only two cases were recorded. In both the membrane was formed only at one end.

Eggs were stratified by centrifugation. As this caused also a slight elongation, the eggs were passing into the capillary with either the light or the heavy pole first. In eight cases the membrane appeared at the inner end which in three of the eggs represented the heavy (Fig. 1 g), in five the light pole (Fig. 1 f). Moreover, one egg raised a membrane at both ends. Thus, the displacement of the interior material in relation to density did not seem to affect the phenomenon described above.

Elongated eggs ejected from the pipette immediately start to round up. This occurs more rapidly and to a fuller extent in unfertilized than in fertilized eggs. The membrane formation was studied in stretched eggs fertilized immediately after being placed under the cover slip. It was noted at which part of the surface the membrane elevation started. Thereby one must be aware of the fact that the membrane takes a spherical shape, whereas the

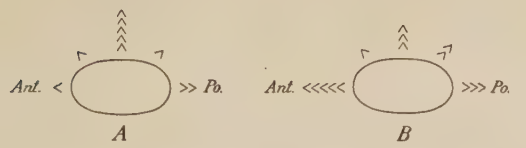

Fig. 2. A. The arrow heads indicate the frequency of the position of the point where the membrane formation initiated in eggs that were fertilized immediately upon expulsion from the capillary.

B. Incidence of breakup of the cortex upon immersion of stretched eggs in fresh water.—Ant., front (anterior) end, Po., hind (posterior) end.

eggs to a varying extent remain elongated. The ends of the egg are thus more closely adjacent to the membrane than the sides. In the majority of cases, the membrane elevation commences along the sides which represent the larger part of the surface. As indicated by the arrow heads in Fig. 2 a, no particular region seems to prevail with respect to the initiation of the process of membrane elevation. The number of cases (10) is, however, admittedly too small. Nevertheless, the data indicate that the release of the egg from the pipette at once removes the inhibition of membrane formation at the sides in contact with the capillary wall and, as a rule, at the hind end as well.

Eggs aspirated in capillaries were ejected into fresh water and their swelling and break-up were studied. In seven out of twenty-one cases, the swelling seemed to be uniform indicating that the surface had remained uniform. In fourteen cases the swelling was more localized, one side bulging out faster until the wall finally burst. The frequency of this phenomenon is shown in Fig. 2 b. The results indicate that the ends are less resistant than the other sides of the egg. There are only three cases of burst on the "long sides," although they represent by far the largest part of the surface. It is also noted that the front end, the normal site of membrane formation in the capillary, seems to be more sensitive than the hind end.

Once the eggs are stretched, their passing up and down in the capillary seems to have no influence on the localization of the membrane. The fertilization membrane presents a double refraction which is negative with respect to the radical direction (10). The retardation amounts to 12–13 mμ (14). The membranes formed at the front or hind ends of an elongated egg are birefringent, and the value of retardation is equal to that of a normal membrane.

The smooth elevated membrane present at one egg pole is continuous with a granular membrane which adheres closely to the egg surface. This granular region may extend over a smaller or greater part of the egg surface, or even cover the whole surface (Fig. 1 h, i, k,). Sometimes one may observe

the sequence: smooth membrane, slightly elevated corrugated or granular membrane, adhering granular membrane, smooth egg surface devoid of membrane.

As a rule the contracted egg surface bulges out under the partial membrane some minutes after fertilization. In this way the egg surface comes in closer contact with the smooth membrane and the vitelline space thus apparently obliterates. In cleavage stages the membrane again becomes more clearly visible.

The surface layer of the mature unfertilized egg exhibits a yellow colour when viewed with dark ground illumination. Following fertilization this layer turns bright white (10). Both in unfertilized and fertilized eggs the luminous layer has a smooth outer surface and a smooth boundary towards the grayish interior cytoplasm. The eggs sucked up into a capillary were also studied with dark ground illumination. The changes were of perfectly normal type in the regions covered with a smooth membrane. However, the part of the egg in contact with the capillary wall behaves differently. Upon fertilization the layer changes also here into white, but it is not quite as sharply delimited and as bright as normally. The outer surface of the layer is slightly irregular. Moreover, the border against the interior cytoplasm is less well defined, and the whole layer gives a more spongy impression than in unfertilized or in normal fertilized eggs. Even if the hind end of the egg has failed to form a partial membrane and presents a smooth surface, it shows a sharply delimited white layer.

The above observations may be summarized as follows. When the membrane is formed only at the front end, the surface changes at this end are normal. The membrane is smooth and birefringent. When passing to the region in contact with the glass capillary, there may be a transitory region with a granular membrane, but the largest part of the surface has here no membrane. When viewed with darkfield illumination, this surface looks more spongy than otherwise. At the hind pole, where the sperm has penetrated, the surface does not show the birefringence typical of the fertilization membrane. If, however, a membrane is formed also at this end, it presents the typical negative birefringence.

In eggs transferred to a drop of sea water under a cover slip, a granular slightly birefringent low membrane was sometimes raised from the granular-spongy surface that had previously been pressed against the wall of the capillary. There is evidently a very short step between the state of the "spongy" surface layer and a true membrane formation.

Eggs with membrane at the front end were ejected into hypotonic or acid

sea water in order to immobilize or kill the sperm. After transfer to normal sea water, spermatozoa were added. No sign of refertilization could be observed.

Eggs with a membrane formed only at the front end are able to develop into plutei.

DISCUSSION

It is a striking feature of the present results that in most cases no membrane is formed at the hind end of the egg, where the spermatozoon has penetrated. In order to discuss this feature in a profitable way, it is necessary to know which changes take place in the process of membrane elevation. The elevation of the vitelline membrane involves an 1.5–2-fold increase of its surface.

According to Runnström (10, 11) and Chambers (1), the elevation of the vitelline membrane is mainly due to forces residing in the elements that constitute the membrane. The most likely explanation is that the protein molecules of the vitelline membrane expand. As is well known, cortical material including the cortical granules merge with the vitelline membrane (7, 12, 15). In this context the discussion may be limited as a first approximation to the behavior of the vitelline membrane. According to available evidence, the elevation of the fertilization membrane seems to be initiated and governed by the expansion of the pre-existing vitelline membrane (11). This latter enters as the outermost part of the final fertilization membrane.

The data presented by Lindahl (4) give valuable indications concerning the different conditions prevailing in the front and hind end of an aspirated egg. He pushed eggs of *Paracentrotus lividus* from the wider part of a pipette into a capillary of the same diameter as that used in the above experiments. Lindahl found that the aspirated eggs break up more easily at the front than at the hind end. Similar observations were made in the course of this work.

Furthermore, Lindahl demonstrated that the red pigment granules of the cortex became less numerous at the anterior end, whereas these granules appeared to be more concentrated at the posterior end of the egg.

Dark field observations showed also that the surface of egg cytoplasm is thinner at the anterior, but thicker and more folded at the posterior end. From this Lindahl (4) inferred that the aspirated eggs are more stretched at the front as compared with the hind end.

Dan and Okazaki (2) also aspirated eggs of several sea urchin species in capillary tubes and studied the effect of this treatment on the birefringence of the surface. Their results on unfertilized eggs of *Heliocidaris* are especially pertinent to the present discussion. An egg was aspirated in a capillary of about the same bore as that utilized in the above experiments. The front end had a round outline and exhibited a relatively strong cortical birefringence. The hind end, on the other hand, assumed a more truncate shape; here no cortical birefringence was apparent. The increased birefringence in the front end is probably due to an increased tension which gives rise to a greater regularity of arrangement of the micellar components of the cortex. Thus it seems that the aspiration induces a certain polarity of the egg, the surface layer of the front pole being more stretched than that of the posterior pole.

At this latter there must occur a slackening or even a compression of the surface layer.

The induced polarity seems to be stabilized in the *Psammechinus* egg, as a consequence of the inward movement occurring when the egg is aspirated into the capillary. No change in the initial conditions follows upon moving the egg up and down the capillary. This is in a certain contradiction to the experience of Dan and Okazaki (2). They found that the polarity with respect to birefringence can be reversed when the direction of the movement of the egg is reversed.

In the present work no systematic observations have been carried out concerning the cortical birefringence, as the attention was mainly concentrated on the membrane formation. A detailed study should be carried out with the view of correlating membrane polarity and birefringence using the same material.

If the eggs are released from the capillary the induced polar differences soon level out at least with respect to the membrane formation (Fig. 2 a). According to Lindahl (4), the aspiration of the egg into a capillary influences its dorsoventral organization. The front side preferentially develops into the ventral (oral) region of the larva. The induced state here persists even after the eggs have resumed their spherical shape subsequent to their release from the capillary.

It seems reasonable that a compressed state prevailing at the posterior end of the aspirated egg would counteract the expansion of the elements of the vitelline membrane and thus also counteract the elevation of the fertilization membrane. In such cases as that represented in Fig. 1 k, the adhering membrane covering the posterior pole of the egg has a granular or folded type. This seems to be in fair agreement with the assumption of a compressed state of the posterior surface, as was clearly demonstrated by Lindahl (4).

The low membrane must be presumed to contain elements that have failed to expand. This may reflect the conditions prevailing at the moment of fertilization. On the whole the fertilization membrane tends to remain in the state which it assumes on fertilization.

The stretching of the surface layer of the front end could have been expected to bring about a maximum expansion of the elements of the vitelline membrane. In this way an elevation of the vitelline membrane would not be possible. The results obtained indicate, however, rather that the expansion of the elements has not been maximal. Beyond the mechanical stretching a spontaneous expansion has occurred as a response to the propagated impulse.

The sides of the egg in contact with the surface of the capillary are also under a certain pressure. If a membrane appears at the posterior egg pole it is smooth and separated from the egg surface. Conversely a membrane appearing in the region adjacent to the capillary wall always adheres to the egg surface and is granular or corrugated.

Not only the membrane elevation is concentrated preferentially to the front end of the aspirated egg, but a contraction of the cytoplasm also occurs. In the present cases the contracted region is not under the direct influence of the spermatozoon, but the contraction is due to the propagation of the impulse engendered at the site of sperm attachment.

As pointed out by Runnström and Wicklund (16) there is a certain correlation between contraction and membrane elevation. The contraction probably occurs in

connection with the expression of substances which bring about the elevation and differentiation of the membrane. These views have been confirmed and further elaborated by Monroy and Runnström (5, 6), and by Hultin *et al.* (3), see also Motomura (8).

It follows from these considerations that the membrane formation must not be regarded as a phenomenon residing only in the most superficial layer of the egg, as was assumed above as a first approximation. The causes of the unipolar membrane formation may also reside in the deeper layers of the cortex. Lindahl (3) found that the front region of the aspirated egg exhibits a lower stability of its colloids than the posterior part. Concurrently with these changes the release of agents such as the Membrane Elevating Factor (MbElF) and the Jelly Precipitating Factor (JePptF), see (3), may be enhanced at the anterior but inhibited at the posterior end of the aspirated egg.

When dealing with the causes of the unipolar membrane formation one has probably to consider both the state of the vitelline membrane and that of the underlying cortex as well.

In the experiments of Runnström and Kriszat (13) the activation was inhibited in the egg region that adhered to the bottom of the glass dish, see above p. 468. The cortical changes occurring upon fertilization involve a rather thorough molecular rearrangement (12). Very likely a strong attachment to a glass surface will block this rearrangement that requires a certain mobility of the components of the cortex. It appears that the contact with the capillary tube has not the same profound effect on the cortex as the attachment to the glass surface in the previous experiments (13). The impulse or wave of molecular rearrangement is here able to pass even through regions where the membrane formation is blocked. This latter process is not a necessary link in the spreading of the activation impulse.

Rather wide perspectives are open before the fact that the front region of aspirated eggs is not only the preferred site of membrane formation. According to the results obtained by Lindahl (3) this region preferentially gives rise to the ventral (oral) side of the larva.

SUMMARY

Unfertilized eggs of *Psammechinus miliaris* were aspirated into capillaries with an inner diameter lesser than that of the egg. As a consequence this assumed an elongated form. The part of the egg which precedes at the aspiration is called the front, the opposite the hind end of the egg. The aspirated egg was fertilized at its hind end. Under these conditions a fertilization membrane appeared at the front end, the membrane formation being inhibited at the hind end and in the part of the egg surface that was in contact with the glass wall of the capillary. In rare cases a membrane was formed also at the hind egg pole. At the contact surface a granular or corrugated membrane may also appear. This membrane adhered to the egg surface.

The results prove that the activation impulse propagates from the site of

sperm entrance at the hind pole to the front pole, although a large intermediate region is incapable of membrane formation.

The phenomenon described is referred to a polarization brought about by the aspiration of the egg, the front end becoming more stretched, the hind end more compressed (Lindahl (4), Dan and Okazaki (2)). The polarization affects probably the vitelline membrane and deeper layers of the cortex as well.

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BRIEF NOTES

ELECTRON MICROGRAPHS OF MYELIN FIGURES AND THE PROBLEM OF SUBMICROSCOPIC NERVE FIBERS

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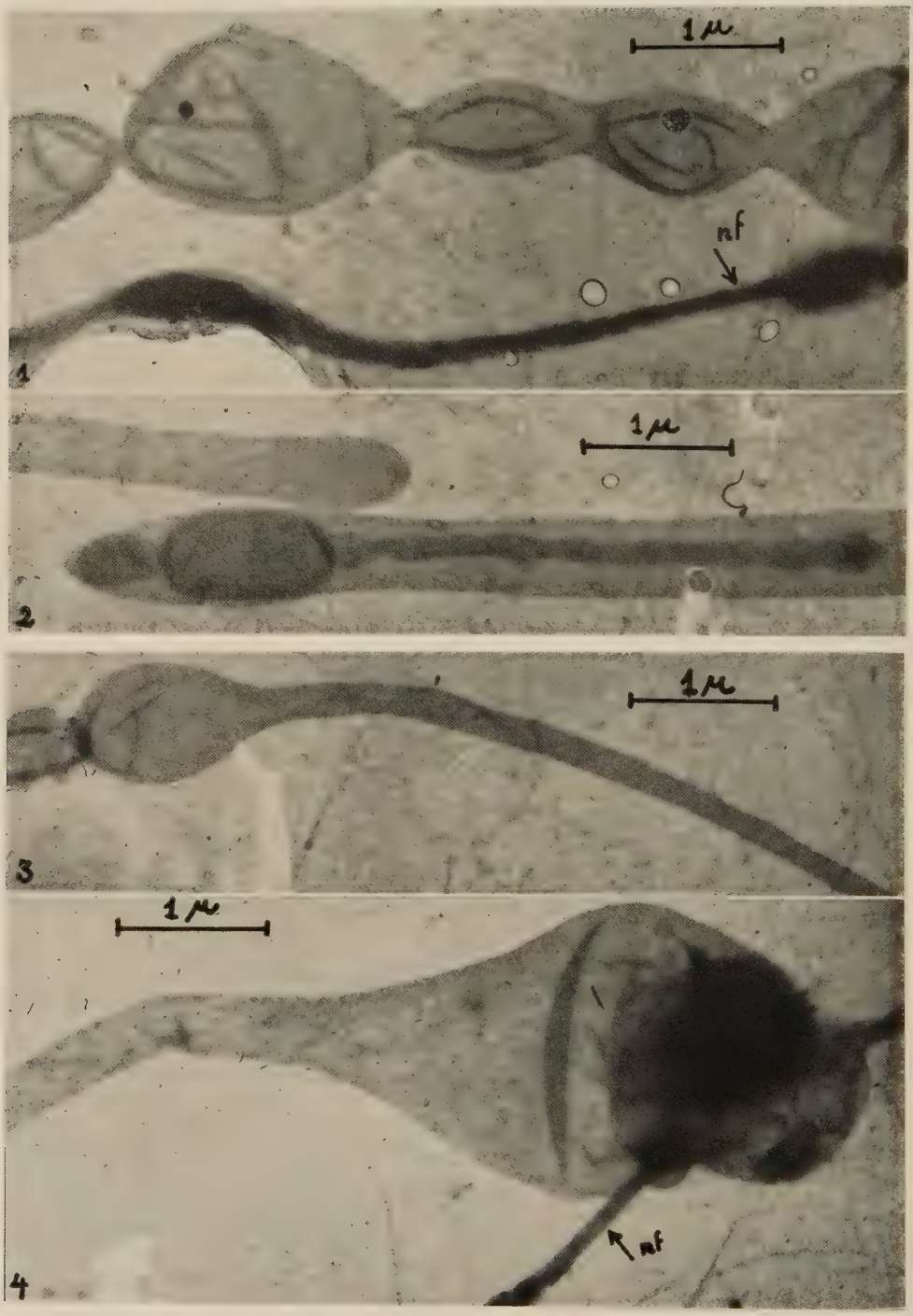
In an electron microscope study of nerve tissue cultured *in vitro* we have recently described nerve fibers ranging in diameter from 0.1 μ up to 1 μ or more (1). A considerable number of nerve fibers, collaterals and terminal tips growing in tissue culture are thus of submicroscopic dimensions and could not be resolved with the optical microscope. In all cases these fibers and collaterals have a rather high electron density and show a compact parallel arrangement of structure within the fiber (see Figs. 8, 9, 10 in (1)). The terminal endings generally show an enlarged mass containing a tightly packed microvesicular material and thin finger-like processes in which a certain orientation and aggregation of this material is seen (see Figs. 11, 12).

In a recent study Fernández-Morán (2) describes fibers, obtained by dissociation of the white matter of the spinal cord, which range in diameter from 0.1 to 0.5 μ, and whose nervous nature is claimed. At variance with the dense submicroscopic nervous fibers grown *in vitro*, these fibers *"merely consist of a single layered tube or sheath containing one filament"* (2). Judging from the published electron micrographs this filament is frequently absent and the entire fiber is reduced to an apparently empty tubular membrane. Some hollow terminal club-like formations of these tubes having about 1 μ in diameter, which according to the author resemble "nerve endings", are also described.

In 1950, in the course of observations of tissue culture material stained with methylene blue we have observed the development of myelin figures which after fixation in osmic acid show a strong resemblance with Fernández-Morán's "fine submicroscopic fibers" and "nerve endings".

Fig. 1 shows a nerve fiber of 170 mμ and a tubular myelin figure provided with several vesicular enlargements. The nerve fiber has a very high electron density and shows a compact parallel structure, while the myelin figure is only an empty tubular membrane with many folds. In Fig. 2 two tubular myelin figures are seen. One of them has some vesicular and tubular structures within it. A wide variety of sizes in the tubes ranging from 100 mμ or less to 1 or more microns can be observed (Figs. 1, 2, 3, 4). The terminal ending of some of these tubes present a club-shaped enlargement (Fig. 4).


1 μ
nf
1
1 μ
2
1 μ
3
1 μ
nf
4

We want to stress the fact that in our tissue culture preparations these myelin formations could always be distinguished from true nerve fibers and nerve endings, and that they were considered as artefacts by us. In view of the similarity of these figures with some of Fernández-Morán's electron micrographs of "fine submicroscopic nerve fibers and nerve endings" the publication of electron micrographs of our artefacts seemed pertinent, since they will probably stimulate interest in the further study of the submicroscopic organization of nerve fibers.

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Fig. 1. Electron micrograph of chick embryo nervous tissue cultured *in vitro* and stained with methylene blue. Below a submicroscopic nerve fiber (*n.f.*); Above a tubular and vesicular myelin figure. Osmic acid fixation. 20 000 ×.
Fig. 2. Electron micrograph showing two myelin figures. The lower one shows myelin vesicles and tubules within the larger tube. Osmic acid fixation. 20 000 ×.
Fig. 3. Tubular myelin figure of about 170 mμ provided of a vesicular enlargement. Osmic acid fixation. 20 000 ×.
Fig. 4. Electron micrograph showing a dense submicroscopic nerve fiber (*n.f.*) of 125 mμ and a tubular myelin figure ending in large club-like enlargement. Osmic acid fixation. 20 000 ×.

OBSERVATIONS ON THE STRUCTURE OF SUBMICROSCOPIC NERVE FIBRES

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Recently De Robertis (1) has called attention to myelin figures observed in nerve tissue cultures, which appear to resemble some of the submicroscopic nerve fibres described in the spinal cord by Fernández-Morán (3). In connection with further studies now being made on the fine structure of these submicroscopic fibres in thin sections and in dissociation preparations, the problem of possible artefacts has also been investigated. The results of these investigations, which will be presented in detail later, confirm the previous findings, making it possible to clearly distinguish the submicroscopic nerve fibres from myelin figures and other preparation artefacts.

Myelin figures of the type shown by De Robertis (1) occur only in poorly fixed material or in preparations of fresh nerve subjected to different manipulations before fixation. Here they can be recognized as relatively short, empty tubular formations, scattered at random, with irregular dilatations following closely upon each other. In contrast, the submicroscopic fibres of the spinal cord (3) are well-defined structures 0.1 to 1 μ in diameter and up to 80–100 μ long, which are found emerging as fine collaterals from the larger myelinated nerve fibres. These fibres consist of a thin sheath formed by a single tubular membrane with fusiform enlargements containing dense bodies. The sheath encloses a varying number of filaments 100–200 A in width. As can be seen in the published micrographs (3) (Figs. 30–45), the fibres always contain internal filaments in parallel array, and all transitions can be found between fibres with 20 or more filaments (Fig. 1) and the finest ones with only one filament (Fig. 2). The finest fibres appear as thin ribbons in the dried preparations, but, with their central filament and the characteristic structures of the membrane at the dilatations (3) (Fig. 41), they are quite different from the empty myelin formations. An "empty" membrane of a submicroscopic fibre was shown earlier (3) (Fig. 37) in order to illustrate the tubular character of the sheath. However, this membrane still contained the adhering dense bodies and exhibited the distinct granular structure which is different from the finer surface structure of the myelin figures. The terminal formations of the submicroscopic fibres invariably contain several round or oval dense bodies (Fig. 45), and can not be mistaken for the empty blebs of the myelin figures.

The submicroscopic fibres described in the spinal cord are therefore essentially similar to the fine nerve fibres demonstrated in nerve tissue cultures by De Robertis and Sotelo (2), except as regards the internal structures. The internal filaments of the submicroscopic nerve fibres (Figs. 1, 2) which appear as "solid" fibrils with distinct contours, are quite different from the double-edged structures described as "neurotubules" by these authors. Upon closer investigation of these structures, it was found that incomplete fixation or slight mechanical agitation of fresh nerve

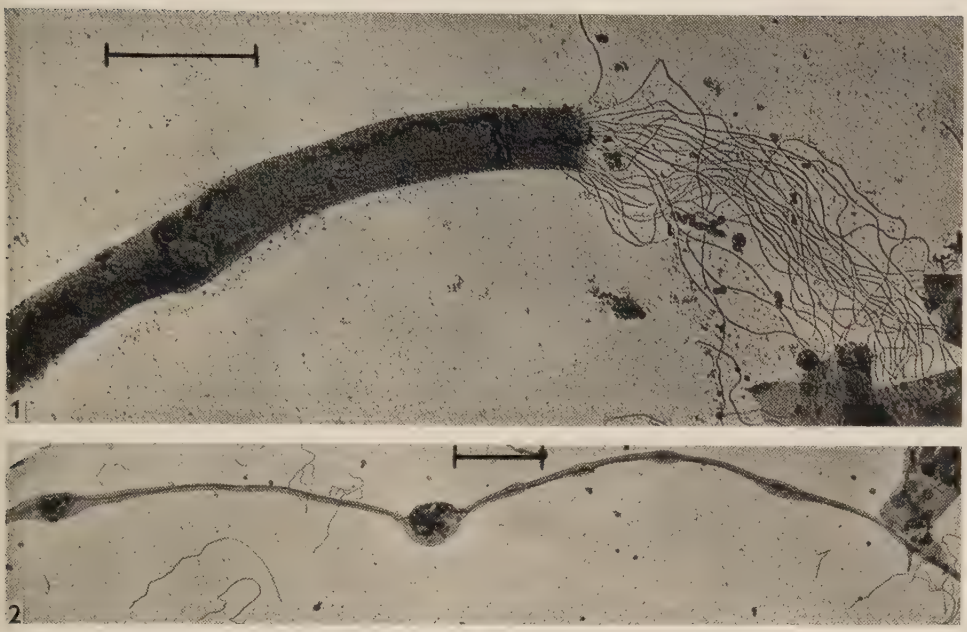

Fig. 1. Submicroscopic nerve fibre of medium size from the osmium fixed lateral funiculus of frog spinal cord. 22 "solid" filaments, approximately 100 A in diameter, are seen emerging from the broken end of the fibre, after coursing in parallel array within the tubular sheath. 20 000 ×.
Fig. 2. Fine submicroscopic nerve fibre isolated after osmium fixation of the whole lateral funiculus of frog spinal cord. This fibre emerged from a larger myelinated nerve fibre and reached a length of over 80 μ. Notice the tubular sheath membrane with dense bodies at the enlargements and the single filament contained within it. 12 000 ×.

preparations before osmium fixation sufficed to modify the appearance of the filaments in a characteristic fashion. The same filaments would then appear as dense-edged "neurotubular" structures in areas contaminated with a diffuse material, and as "solid" fibrils with distinct contours in an uncontaminated portion. The contaminating material adheres intimately to the filament, forming a diffuse covering which is different from the well-defined, wide tubular membrane of the submicroscopic fibres. Moreover, the spurious "neurotubular" appearence can be abolished by local application of a droplet of alcohol-ether which dissolves the contaminating layer. In thin transverse sections of the spinal cord the bundles of submicroscopic nerve fibres can be recognized as closely packed rings containing the distinctly outlined, dense axon filaments.

Considering the variety of artefacts which can arise when analysing the labile submicroscopic structure of nerve tissue, it is therefore important to evaluate the findings critically, as pointed out by De Robertis.

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OBSERVATIONS SUPPORTING THE PRESENCE OF A NUCLEAR UPTAKE OF S^{35}-LABELED SULFATE IN THE MOUSE

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Radioactive sulfate has been shown to enter rapidly (within one day) into the nuclei in the root meristem of the plant *Vicia faba* (8, 5). In the animal organism, S^{35}, given as sulfate, has mainly been found in sulfo-mucopolysaccharides (1, 7) or in ethereal linkage (6). It does not seem to be directly utilized in the synthesis of sulfur-containing amino acids (9, 2). In the present experiment, S^{35}-labeled sulfate was found to be fixed in the nuclei as visualized with autoradiography.

Experimental. Fifteen adult mice were given subcutaneous injections of carrier-free S^{35}-labeled sodium sulfate (supplied by A.E.R.E., Harwell, England) in amounts of 60 μC/g of body weight. Five mice were sacrificed after 48 hours and 10 after 21 days. The ovaries, uteri and parts of the intestines were fixed in methanol for 24 hours, passed through isopropanol for 3 hours, embedded in paraffin and cut in 4 μ thick sections, which were mounted on glass slides and deparaffinized. Stripping film autoradiographs were prepared (4). The exposure time amounted to 24–40 days.

Results. After 48 hours no apparent nuclear accumulation of S^{35} could be detected; it might, however, have been masked by a more intense general accumulation of S^{35} in many tissues. After 21 days there was a special nuclear accumulation of S^{35} as illustrated by Fig. 1 and Table I. The nuclei were identified in the phase contrast microscope.

Discussion. As far as we know, the sulfate ion is mainly utilized for the synthesis of esterified compounds in the animal organism. A compound of the sulfo-mucopolysaccharide type, the sulfo-lipid type or of a similar nature could therefore be expected to exist in the cell nuclei. An interesting parallel to this supposition is a recent report by Bychkow *et al.* (3). On the basis of estimations of the total N to hexosamine N ratio in several animal tissues, these authors suggest the presence of mucopolysaccharides or of mucoproteins in several types of cell nuclei.

The possibility that S^{35} occurs in other sulfur-containing compounds than mucopolysaccharides in the nuclei cannot be excluded by the present investigation. The retention of considerable amounts of inorganic sulfate as long as three weeks after the injection is, however, highly improbable; nor do the sulfur-containing amino acids seem to be responsible for the high S^{35}-uptake in the nuclei (2, 4).

Summary. Autoradiography has shown that radioactive sulfur is incorporated in the cell nuclei of some tissues in mice. The S^{35} was administered as sulfate. The nature of this incorporation is still obscure, but the presence of a nuclear sulfo-mucopolysaccharide seems probable.

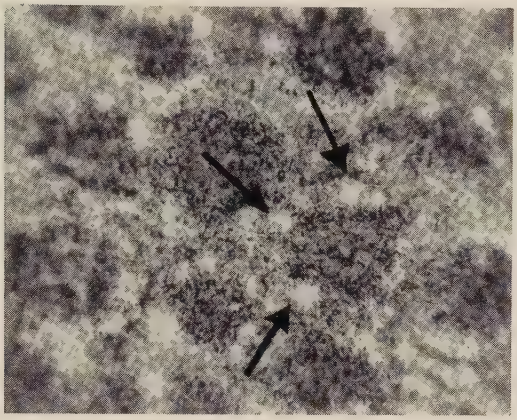

Fig. 1. Stripping film autoradiograph of intestinal epithelium of mice, showing nuclear accumulation of S^{35} 21 days after an injection of S^{35}-labeled sodium sulfate. (× 350.) The arrows point to the nuclei which appear white (positive print).

TABLE I

Tissue	Nuclear upptake of S^{35}
Endometrial epithelium	(+)
Endometrial stroma	+ + to + + +
Myometrium	—
Subperitoneal tissue of uterus	+
Ovarian connective tissue	+ to + + +
Granulosa tissue (radiation-damaged)	(+)
Young corpora lutea	+
Egg cells	—
Intestinal epithelium 1	+ + to + + +
Intestinal submucosa	+ +
Intestinal muscle layer	(+)

In the table, — denotes no S^{35}, +, + +, + + + denote small, moderate and large amounts, respectively, of S^{35}.

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MICRO X-RAY DIFFRACTION IN HISTOCHEMISTRY

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The aim of histo- and cytochemical studies is to identify and eventually to quantitate substances on a microscopic level. This necessitates isolating extremely small areas for analysis, preferably in the intact biological tissue. Physical methods have proven to be of value in this respect, in particular the radiant energy microabsorption techniques. In addition, X-ray diffraction methods can now be applied at this microscopic level of investigation.

The great importance of X-ray diffraction methods in studying biological systems is well illustrated by the results obtained from essentially homogeneous macroscopic samples. Information concerning molecular orientation, particle and molecular sizes, and in certain cases detailed molecular structure has been obtained.

Micro-X-ray diffraction methods can be applied to certain histochemical problems, but with some limitations as compared with diffraction studies in the macroscale.

There are certain respects in which microdiffraction is particularly useful when applied to biological problems. In systems in which changes in structure occur over short distances it is often possible to follow this change in detail by a series of microdiffraction photographs. An example of this is the process of formation of keratin in skin and similar tissues. Another example is the study of individual Haversian systems in bone tissue (1). In such cases an overall picture could be obtained by macro X-ray diffraction, but important additional information is obtained by studying the variation in the biological system by microdiffraction.

In systems where occlusions occur and it is necessary to isolate the diffraction pattern of the occlusion from that of the surrounding material, the micro beam techniques can often be applied. Thus, diffraction patterns of small crystalline areas have been recorded (6, 7). Diffraction patterns of small occlusions in experimental urinary calculi have been valuable when studying the genesis of these calculi.

The emphasis has usually been on the wide angle pattern, as this is the more readily examined with present microdiffraction apparatus. The practice is to use a fine capillary (3) or a very small pinhole as a collimator. The sample to film distance is small because of the low intensity and the divergence of the beam. The divergence from a small pinhole is such that a sample to film distance of only a few millimeters can be used, and spacings longer than about 10 Å cannot be resolved. In the case of the fine capillary the divergence is not so great, and specimen to film distances can be increased so as to record diffraction spacings up to about 60 Å. Total reflection from the glass capillary begins to interfere in this region. The diffraction bands are not very sharp, and closely spaced lines cannot be resolved. Fig. 1 c shows that the 43 Å ring of a nerve specimen can be resolved, but the equatorially orientated bands at higher spacings are not separated. Bergmann and Fankuchen (2) report having

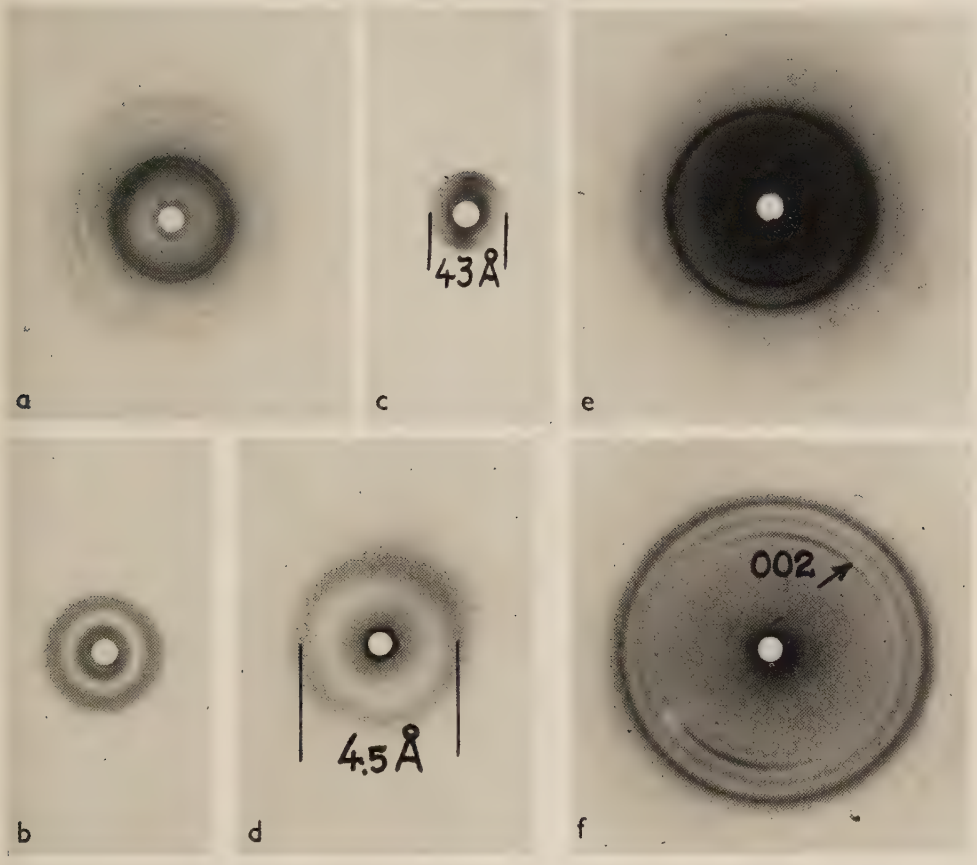

Fig. 1 a and b: Wide angle patterns of two closely situated areas in the hoof of a new born calf.
1 c and d: Low angle and wide angle patterns of dried nerve. In the low angle diagram the totally reflected primary beam can be seen close to the center.
1 e: Wide angle diagram of a thin layer of dentine in the root tip of a growing guinea pig tooth. The pattern is that of hydroxylapatite.
1 f: Wide angle pattern of a single Haversian system in a heated section of cow femur showing orientation in the 002-ring which has the spacing 3.44 Å.

modified such a microdiffraction camera in order to resolve spacings up to 100 Å. Although the long spacing diffraction bands recorded in the microcamera are not very well defined, nevertheless they are associated with a very small area of the sample, and can readily be correlated with the wide angle pattern from the same area.

There appear to be several possible ways in which the micro diffraction method might be refined for precise histochemical work. The development of the X-ray microscope (5) may provide a means for producing very fine X-ray beams. Thus it may be possible to obtain diffraction patterns from areas smaller than 1 micron

square. It also seems important to make a precise study of the low angle diffraction from small areas in biological systems, and this may be achieved by using simple aperature collimation on a microscale.

Finally it may be of advantage in many cases (e.g. particle size determination from the diffuse low angel scatter (4)) to vary the type of radiation used. Thus when AlKα is used instead of CuKα the scattering power can be considerably increased, and the required thickness of the sample may be reduced. Further experiments along the lines mentioned are in progress.

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OBSERVATIONS ON ULTRA-CENTRIFUGING OF HUMAN RED CELL GHOSTS

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A polarised light analysis, (1, 2), has shown that the membrane of the human red cell ghost is about 0.5 μ in thickness, which is relatively large compared to earlier estimates. The experiments described below were done in order to try and confirm this result by another method. They were carried out, and mentioned in a preliminary note (1), before this writer knew of the similar experiments and results of Ponder (4), but they are worth publishing both as a confirmation of Ponder's results and because higher speeds of centrifuging were used.

Preliminary experiments with a low speed centrifuge (8000 g) confirmed the finding of Ponder (4) that human ghosts pack down in a centrifuge to give a layer of about the same height as the equivalent number of red cells. If the ghosts were closely packed, the volume of each ghost would have been on an average 90 μ^3.

The main experiment was done in order to find the variation of ghost volume with speed, and also to see the effect of very high speeds. This was carried out with Dr. R. Markham at the Molteno Institute, Cambridge, an air driven ultra-centrifuge being used in which the cell could be observed, and the height of the layer of ghosts measured, while the rotor was in motion.

Ghosts were prepared by taking fresh citrated human blood, washing the red cells twice in saline, and then haemolysing them by adding ten volumes of distilled water. The resulting ghosts were centrifuged at low speed in order to give a concentrated suspension. A haemocytometer count gave one ghost in every 113 μ^3 of the suspension. The suspension was then placed in the centrifuge cell and the rotor started. It reached a speed of 20000 rpm. (29000 g) after about 15 minutes, and was then kept at this speed for another 15 minutes after which a reading was taken of the heights of the ghost layer and the total cell contents. The speed was then increased to 30000 rpm. (65500 g) and another reading taken after 5 minutes at this speed. This was repeated at 40000 rpm. (116000 g), after which the speed was reduced readings being taken every 10000 rpm. down to zero. The whole experiment from start to stop lasted 80 minutes. The results are given in Table I, with close packing of the ghosts being assumed in the last column.

The results above give the volume of the human ghost as 81 to 75 μ^3, if the ghosts are assumed to be packed tight. Also there is little alteration of the volume on increasing the force of centrifuging up to 116000 g. These figures for the ghost volume are nearly the same as that for the volume of the intact red cell (volumes by different workers vary from 82 to 95 μ^3 (3)).

Table I

	(1) Centrifuge speed in rpm.	(2) Centrifugal 'force' in g	(3) Vol. of ghost layer as per cent of contents	(4) Vol. of a ghost in μ^3
Increasing.....	20 000	29 000	71.5	81
	30 000	65 500	70	79
	40 000	116 000	66	75
Decreasing	30 000	65 000	67	76
	20 000	29 000	66.5	75
	10 000	7 300	67	76
	0	0	68	77

The values for the ghost volume depend on the unproved assumption that the ghosts are closely packed in the layer at the bottom of the centrifuge cell, and that there is no water between them. The experiments of Ponder (4) are more complete because he has been able to find the concentration of ghosts in such a layer by conductivity measurements. He shows that the ghosts are not closely packed after centrifuging at 20000 g, and that the ratio of ghost volume to red cell volume is not 1 but varies from 0.5 to 0.25 with increasing volumes of water used for the initial haemolysis (though he points out that 0.25 is a minimum value). This would seem to indicate that the ghost volumes given above are too high and should be reduced by a factor of from 2 to 4.

It is uncertain, however, how far the results from Ponder's experiments can be applied to these experiments, since the ghosts in this case were centrifuged in water (or more strictly in blood diluted ten times) whereas Ponders's ghosts were in saline. It also seems improbable that if the ghost layer contained from 50 to 75 per cent of water, it would stay at approximately the same height through the wide range of speeds in the ultracentrifuge experiment.

However, even if the volume of the ghost were as little as one quarter of that of the intact cell, these results would still agree with the hypothesis of a relatively thick membrane. If the ghost membrane is 0.5 μ thick, its volume will be nearly 50 μ^3 or about half the volume of the intact red cell. The volume of the packed ghost will therefore be of the same order as the volume found in these centrifuging experiments, if the ghost is simply a collapsed membrane 0.5 μ in thickness. On the other hand, if the ghost were a thin-walled bag with a fluid interior, it might well be expected to collapse under powerful centrifuging and have a final volume very much less than that of the intact red cell.

These results are not, of course, an adequate proof of the thickness of the ghost membrane. The ghost might maintain roughly the same volume as that of the intact cell if it had a thin membrane kept in shape by its inherent strength. It seems unlikely, however, that a cell membrane would have sufficient strength for this. Sea

urchin eggs, for example, collapse into thin flat discs when centrifuged at a few thousand g. It seems much more convincing to assume that the ghost has a thick membrane which simply collapses flat on centrifuging.

It should be added that the word "membrane" is used in this paper to include both the thin permeability membrane of lipids or lipoproteins at the cell surface and also the much thicker structural membrane within. The structural membrane probably resembles the "ultrastructure" suggested by Ponder and others. This concept, however, is discussed more fully in another paper (2).

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A MODIFICATION OF THE WATCH GLASS TECHNIQUE FOR THE CULTIVATION OF ENDOCRINE GLANDS OF INFANTILE RATS

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By introducing a simple modification in the original watch glass technique of Dr. Fell (1) we have succeeded in keeping a number of whole or halved endocrine glands of infantile rats in a healthy state for unusually long periods.

Those familiar with the "classical" methods of cultivation, know that some complex tissues or small organs or parts thereof, in order to be able to survive for more than a few days must be mounted on the surface of the plasma clot. In other words the explant must not be allowed to sink deeper into the medium. Unfortunately, this is what normally happens with most explants, for they all are capable, in a greater or less measure, of liquefying the plasma clot. Placing the explant on the surface of the plasma clot, however, is not an ideal solution of the problem of assuring the gaseous medium to the explanted organ under all circumstances, as much of the explant remains exposed to the atmospheric air. The exposed surface dries if liquefaction of the clot proceeds slowly which is, usually, the case with most organ explants after prolonged cultivation.

The tendency of tissue cells to migrate actively when in contact with solid surfaces is another well known fact (4), young cells being more susceptible to it than those more advanced in age. With organ cultures migration of cells from the mother explant is highly undesirable since it causes a gradual dissipation of the explanted organ, a disruption of its original histological picture and may result in a direct exposure of the parenchyma to the action of the culture medium which, as far as we know, is always injurious to it.

To avoid these and some other technical difficulties, which prevent the worker from making full use of the possibilities that the nutrient medium offers for the survival of the explanted gland and the preservation of its original pattern, we place the explants on a series of glass rods in the following way:

Four or five rods about 100 micra in diameter and 3 cm in length are placed alongside one another on the surface of the coagulum. Once there the rods will not sink in the clot or cause excessive fluid medium to accumulate along their surface. A free space should be allowed between every two neighbouring rods to permit the explant to act upon the clot lying below. Placed on glass rods the explant can be kept submerged to any desired depth in the fluid exuded from the clot in two ways: by allowing a greater distance between two consecutive rods, and by regulating the amount of the fluid medium which can be forced to the sur-

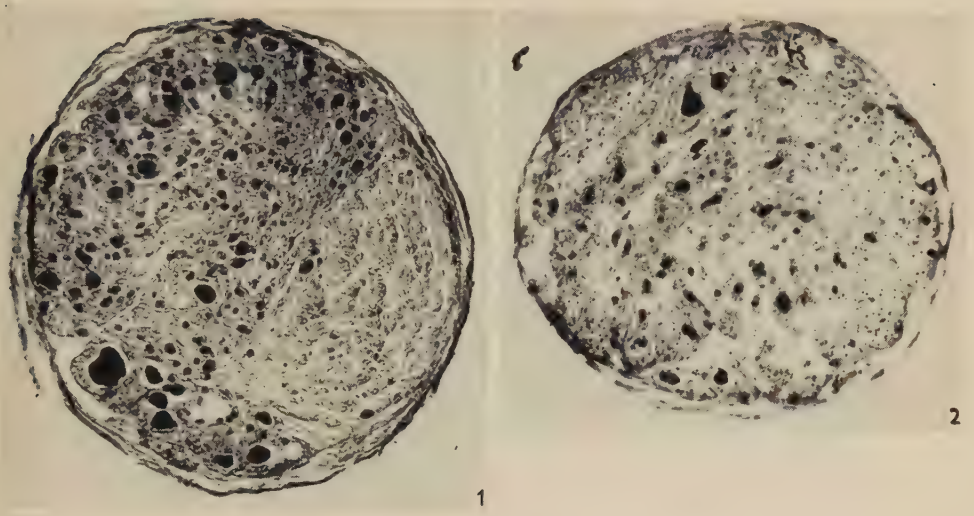

Fig. 1. Thyroid-parathyroid complex of a 17 days old rat after 73 days of *in vitro* life. ×150.
Fig. 2. Thyroid gland of a 17 days old rat after 115 days of cultivation. ×150.

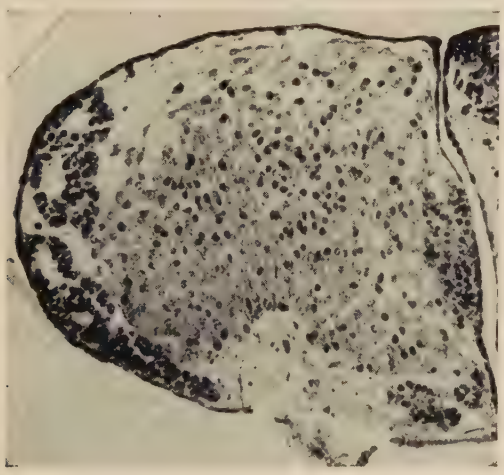

Fig. 3. Posterior pituitary of a 30 days old rat 23 days *in vitro*. Note: Groups of cells of pars intermedia scattered along the periphery. ×130.

face by moving a fine glass rod around the explant without applying any extra pressure. The same desired objective is attained if peripheral rods are slightly thicker. In this way, lengthwise to the rods a central trough is formed allowing the explant to sink in the fluid medium to the depth desired. By shifting the explant with a fine platinum spatula or by pushing it forward once or twice daily, prefer-

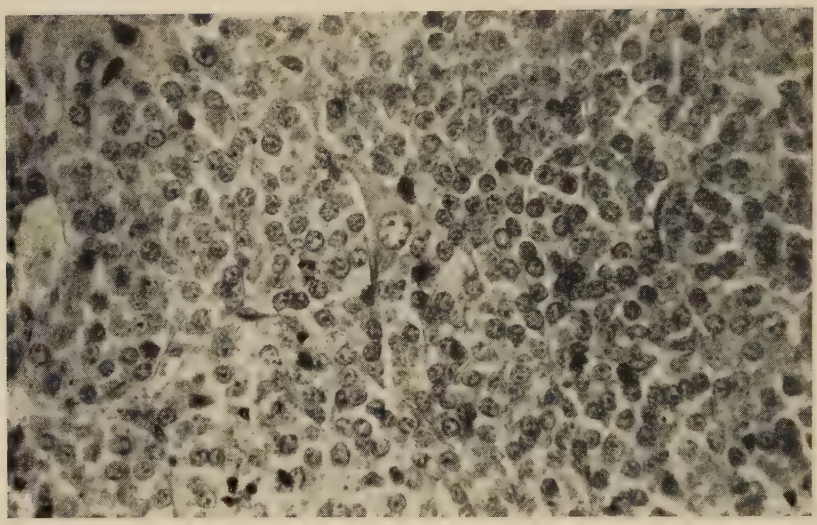

Fig. 4. Adrenal medulla of a 5 days old rat after 22 days of cultivation. Note: A large ganglionic cell in the center of the field, and a small blood vessel on the left. × 350.

ably with a blunt glass needle, along the supporting rods to a new position, an operation that can be repeated indefinitely, cellular migration is effectively prevented and the explant is brought in contact with fresh medium.

All these operations require some experience and dexterity of hand, and must be carried out under a disecting microscope (× 12), for practically every endocrine gland shows, in relation to the medium in which it lives, peculiarities of its own. We usually place two rows of rods on the surface of the clot in the same watch glass leaving one series of them in the reserve.

The advantages gained by cultivating endocrine glands of infantile rats on glass rods in watch glass cultures are: 1) No migration of cells resulting in a gradual dwindling of the original explant takes place; 2) The external capsule serving as an effective shield to the parenchyma cells lying below remains intact; 3) If the explant is not too bulky, central necrosis is prevented; 4) The original pattern of the tissue is more effectively preserved; 5) The results of cultivation are quite uniform.

Cultivated in this manner, infantile thyroid glands have been kept in a healthy state for 115 days (Fig. 1), parathyroids 73 days (Fig. 2), anterior pituitaries 3 months, posterior pituitaries 23 days (Fig. 3), and the adrenal medulla 22 days (Fig. 4). Cultivating the adrenal gland on glass rods helps the medullary portion of the organ, but not the cortex.

The medium used for the cultivation of all above mentioned glands, which was changed once a week, was composed of rat plasma: 7 parts, chicken plasma: 2 parts and chicken embryo extract in 25 per cent concentration: 8 to 10 parts. Incubating temperature: 32° C.

Note: We should like to mention in passing that culturing on glass rods in watch glasses in some ways resembles the roller tube technique of Dr. Gey (2, 3), for in both cases the explants are well exposed to the gases of the atmosphere, while the fluid medium in which they are bathed is never allowed to become stagnant. About these similarities of the two cultural techniques, however, more will be said upon a later occasion.

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A SIMPLIFIED METHOD FOR THE PREPARATION OF THE REFERENCE SYSTEM USED IN X-RAY MICRORADIOGRAPHY

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X-RAY microradiography used in connection with extraction and enzyme digestion has been developed for quantitative determination of substances of biological importance in single cells (2).

In the determination the various fractions are obtained as differences in mass expressed in $mg \cdot 10^{9}/\mu^3$. Frozen-dried sections of cells are exposed at 8–10 Å together with a reference system with the same wave-length dependence. The mass per surface unit of the reference system is supposed to be known and the mass of the cell structure can thus also be determined from the transmission values of the radiogram. It is obvious that the accuracy of the method is dependent upon the accuracy with which the mass of the reference system can be determined.

In 1951 Brattgård and this writer (1) made an investigation to find out what actually takes place from a physico-chemical viewpoint in the production of 2–8 μ thick nitrocellulose films for reference system, and also gave a description of an apparatus for producing such films. The method was founded on weighing in order to determine the mass per surface unit of the film. A certain area of films was weighed on a microbalance, while another part was used for the preparation of the reference system. This required a uniform thickness inside different parts of the film. In order to utilize this the apparatus had to be rather complicated.

Formerly it had been taken for granted that a cellulose film of the thickness in question, which is formed on a water surface without any control, is of uniform thickness. The investigation of Brattgård and this author showed that such a film may vary in thickness even within a rather limited area up to 70 per cent.

Later on, Ingelstam and the author (4) showed that the thickness of a nitrocellulose film can be determined within a small area by means of interferometric measurement. The mass per surface unit can then be obtained by means of the specific gravity of dry nitrocellulose. An interferometer for this purpose has been constructed by Djurle and the author (3) making it possible to measure the thickness of the various steps in the reference system in a simplified manner with an accuracy of $\pm$ 2 per cent. As the result of using this method, it is no longer necessary to determine the mass per surface unit within any other part of the film than that used for the production of the reference system. It is therefore not essential that the film has the same uniform thickness within a large region, and this permits the apparatus described by Brattgård and the author to be constructed in a less complicated way (Figs. 1 and 2).

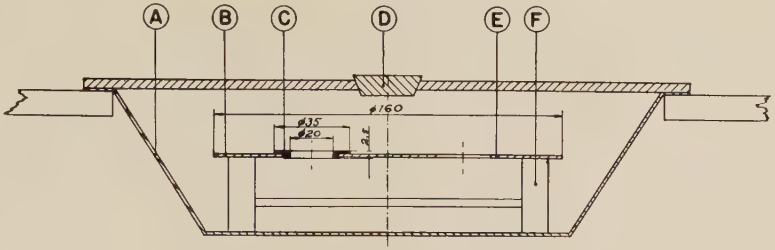

Fig. 1.

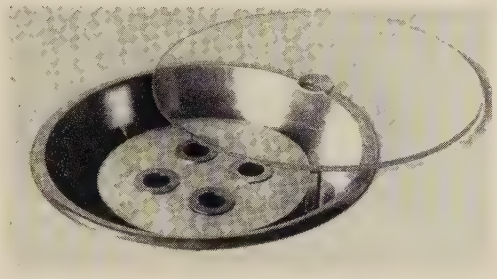

Fig. 2.

The apparatus consists of a round vessel A with a lid D. In the vessel there is a supporting plane B held up by a stand F and equipped with four circular holes in which cylinders C are placed. The vessel is filled with water and this water reaches just over the supporting plane. Nitrocellulose is dropped through the hole in the middle of the lid and spreads itself on the water surface. Subsequently the lid is raised a few centimeters and the film is allowed to dry until it begins to shrink round the circumference. Then the film is caught by lifting the supporting plane after which it is allowed to dry itself fast to the cylinders in dust-free air. The cylinders with the fastened film is detached from the supporting plane and used for the production of the reference system under a microscope.

Compared with the earlier method for the elaboration of the reference system the description given above shows a considerable simplification of the apparatus as well as the procedure.

Interferometric measurements have shown that variations in thickness in the film do not occur within such small areas as the different steps in the reference system. The film fulfills therefore the demand made upon it.

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ASPECT DE LA SUBSTANCE DE NISSL AU MICROSCOPE ÉLECTRONIQUE

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Les corps de Nissl représentent indiscutablement un des exemples les plus caractéristiques de substance basophile cytoplasmique.

En effet, les résultats obtenus par les méthodes spectrophotométriques de même que les procédés basés sur la digestion par la ribonucléase ont montré qu'ils sont constitués essentiellement par des ribonucléoprotéides au même titre que les substances basophiles cytoplasmiques d'autres cellules (5, 6). Or, au microscope électronique les éléments vecteurs de la basophilie cytoplasmique sont bien visibles et ont une structure filamenteuse très particulière, comme nous l'avons montré dans le foie, le pancréas et les glandes salivaires (3). Ils correspondent à l'ergastoplasme des anciens auteurs.

Il parassait donc intéressant de voir si les corps de Nissl présentent une structure analogue et une telle étude nous semblait d'autant plus indiquée que Pease et Baker (8) n'avaient pas réussi à les voir au microscope électronique autrement qu'avec des procédés de fixation qui grossissent la trame (Flemming, Zenker, Formol) et en avaient conclu qu'ils correspondaient probablement à un artéfact.

Les cellules de ganglions spinaux de lapin ont été choisies comme matériel d'étude.

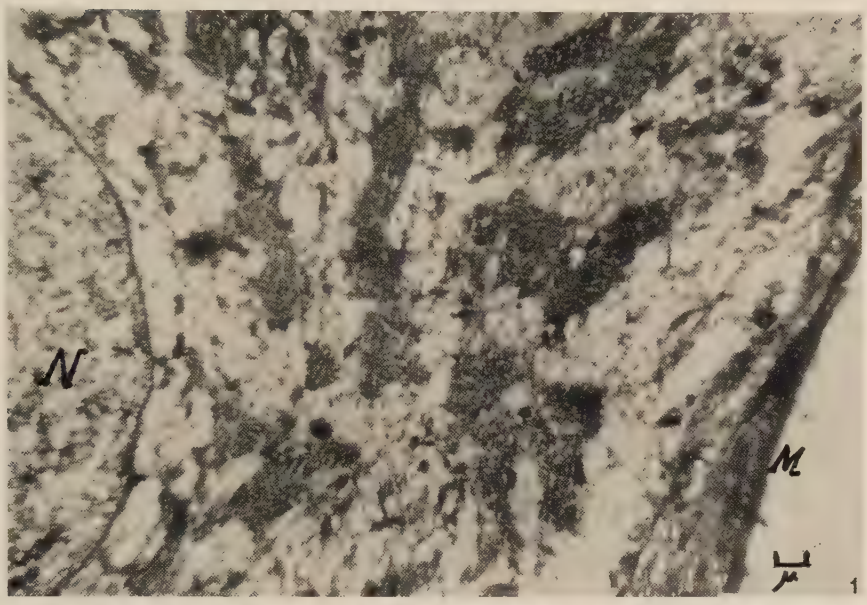
La *fixation* a été obtenue par une injection intra-rachidienne sous-occipitale d'acide osmique à 1 % dissous dans du Ringer sur l'animal anésthésié au Pentothal suivie d'une saignée puis d'une nouvelle injection intra-rachidienne *in situ* et enfin d'une immersion du ganglion prélevé dans l'acide osmique à 2 % (Ringer), l'ensemble de ces opérations n'excédant pas 5 à 7 minutes. Durée totale de la fixation: 4–5 h.

C'est en prenant les précautions préalables indiquées que l'on obtient les meilleurs résultats et que les images sont assez fines pour que l'on puisse exclure la possibilité d'artéfacts.

En effet, les essais de prélèvement sans injection intrarachidienne, ou le choix d'un matériel qui ne permet pas ces manœuvres (ganglion spinal humain, cellules de la corne antérieure de la moelle chez le cheval) nous ont donné des résultats grossiers en comparaison de ceux-ci.

Enfin l'emploi de solutions *tamponnées* d'acide osmique explique certainement aussi la différence entre nos résultats et ceux de Pease et Baker.

L'inclusion et les *coupes ultrafines* (0,1 μ–0,2 μ) ont été effectuées selon une méthode décrite antérieurement (5). (Inclusion à l'Esterwax, coupes à l'ultramicrotome Rocking.) Cette méthode a été employée même pour les contrôles optiques (0,8 μ) ce qui a permis de faire une coloration de Nissl sur le même bloc malgré la fixation osmiée.



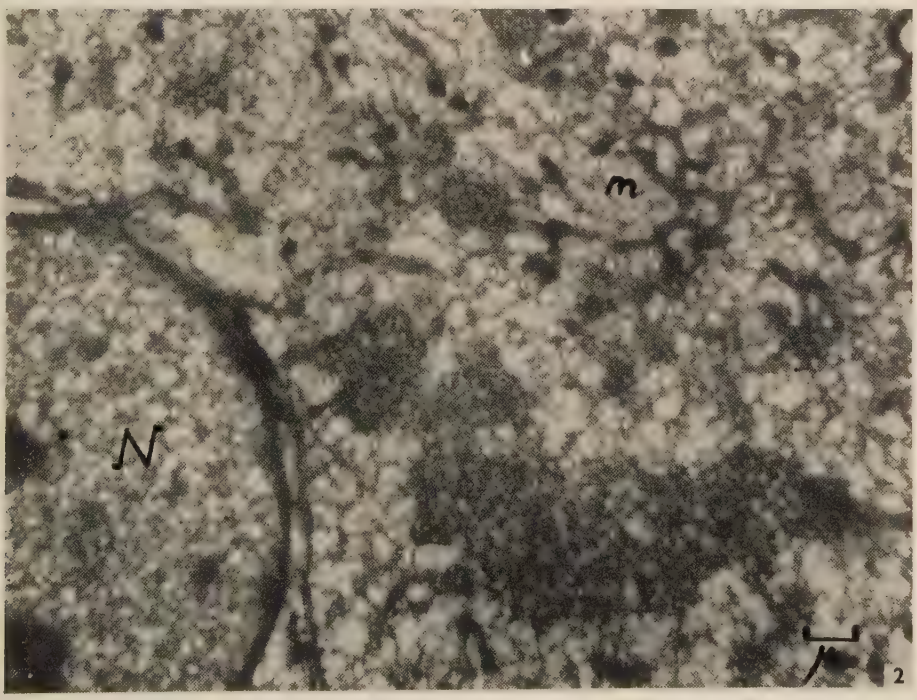


Fig. 1. Portion de cellule de ganglion cérébro-spinal de lapin. A gauche le noyau (N) à droite la membrane cellulaire (M). Disséminés sans ordre au sein du cytoplasme plusieurs amas de substance de Nissl. — Gross. × 430.

Fig. 2. Portion de cellule de ganglion cérébro-spinal de lapin. Le noyau visible (N) n'appartient pas à la cellule ganglionnaire, mais à une cellule satellite. Dans le cytoplasme, au dessus d'un gros amas de substance de Nissl on voit bien les mitochondries (m). — Gross. × 74.

Les figures 1 et 2 montrent dans les cellules ganglionnaires une répartition des corps de Nissl en tous points comparable à celle des contrôles optiques.

Cette répartition classique pour les cellules ganglionnaires est du type «gryochrome» c'est à dire que les corps de Nissl sont disposés en amas granuleux isolés sans orientation déterminée.

On trouve en effet disséminés au hasard dans la cellule, des plages de matière dense, de forme absolument irrégulière et de tailles diverses. Elles se détachent avec netteté du cytoplasme environnant qui apparaît transparent par rapport à elles. Ces amas ne ressemblent en rien, tout au moins au premier abord à l'ergastoplasme filamenteux mis en évidence ailleurs (3). Il s'agit ici de granulations fines denses, serrées les unes contre les autres de l'ordre de 70 à 140 mμ.

Un examen attentif de ces plages permet de retrouver souvent une ordination de ces grains qui deviennent alors de véritables petites fibrilles courtes, disposés soit parallèlement soit en un très fin réticulum. Cette disposition est exagérée avec des fixations plus grossières qui épaississent la trame (Formol-Alcool, Formol neutre à 4 %).

Entre corps de Nissl et *mitochondries* il ne semble pas y avoir de rapports particuliers, tout au moins dans ces conditions expérimentales. Les mitochondries parfois nombreuses sont disséminées au hasard entre les plages de substance de Nissl.

Cette méthode ne permet pas non plus de voir de *neurofibrilles;* nous n'entrerons pas ici dans une discussion à ce sujet. Quant aux *nucléoles*, dont on sait le rôle essentiel que les auteurs modernes lui font jouer dans l'élaboration de la substance de Nissl (1, 7, 9); chaque fois que la coupe les a intéressés ils étaient volumineux et leur structure était nettement filamenteuse comme celle que nous avons pu montrer au niveau d'autres organes (4).

Pour conclure on soulignera la parfaite concordance entre les images classiques de la substance de Nissl et celles révélées par le microscope électronique. Quant à son ultrastructure, elle diffère de l'ergastoplasme des cellules glandulaires.

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THE SIZE OF SPONTANEOUS MOUSE ENCEPHALOMYELITIS VIRUS STRAIN FA, AS DETERMINED BY ELECTRON MICROSCOPY

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The diameter of the spherical particles of spontaneous mouse encephalomyelitis virus was reported by Leyon and Gard in 1950 (2) as approximately 28 mμ from electron microscopical measurements. This value was obtained by measurement of rows of aggregated particles in electron micrographs of a purified preparation of the virus, which method was found to give more reproducible values than measurements of individual particles. Micrographs obtained later (1) were recently copied on paper at a considerably greater enlargement than earlier. The particles then appeared somewhat deformed and especially did the ones in the rows show flattened contact surfaces. The diameter of the particles as determined by a measurement of several particles lying pressed against each other may thus be assumed too small.

A measurement of particle size undertaken on the enlarged paper micrographs gave the diameter of the particle as 30.2 ± 0.5 mμ for free-lying individual particles and 26.9 ± 0.7 mμ for particles in rows of 3—7.

Further it has been observed on several occasions that the mode of drying the particles before the shadow-casting may have an effect on the appearance of the particles in the electron micrographs. The measurements reported (2) were made on particles which had been treated, during the purification procedure, with a strong salt solution and ether, frozen and thawed and kept at $+4°$ over night in 0.001 M phosphate buffer solution. Immediately before the shadow-casting, this solution was diluted with distilled water and drops were placed on the collodion membranes. The water of the drops was evaporated during a few minutes of warming under an ordinary electric illuminating lamp. Thus, with this method the particles were very likely to be heat-coagulated. To avoid aggregation the collodion membranes were often covered with a thin layer of beryllium (Fig. 1). In such preparations the particles appear approximately spherical and do not seem much deformed.

A method of drying the suspension-drops over phosphorous pentoxide was also tried. In all micrographs of preparations treated in this way, however, the particles look rather much deformed. They appear to be shrunk and also to consist of smaller units. The size of these smaller units is difficult to measure in the micrographs but it may be estimated to about one fifth of the diameter of the whole particle (Fig. 2).

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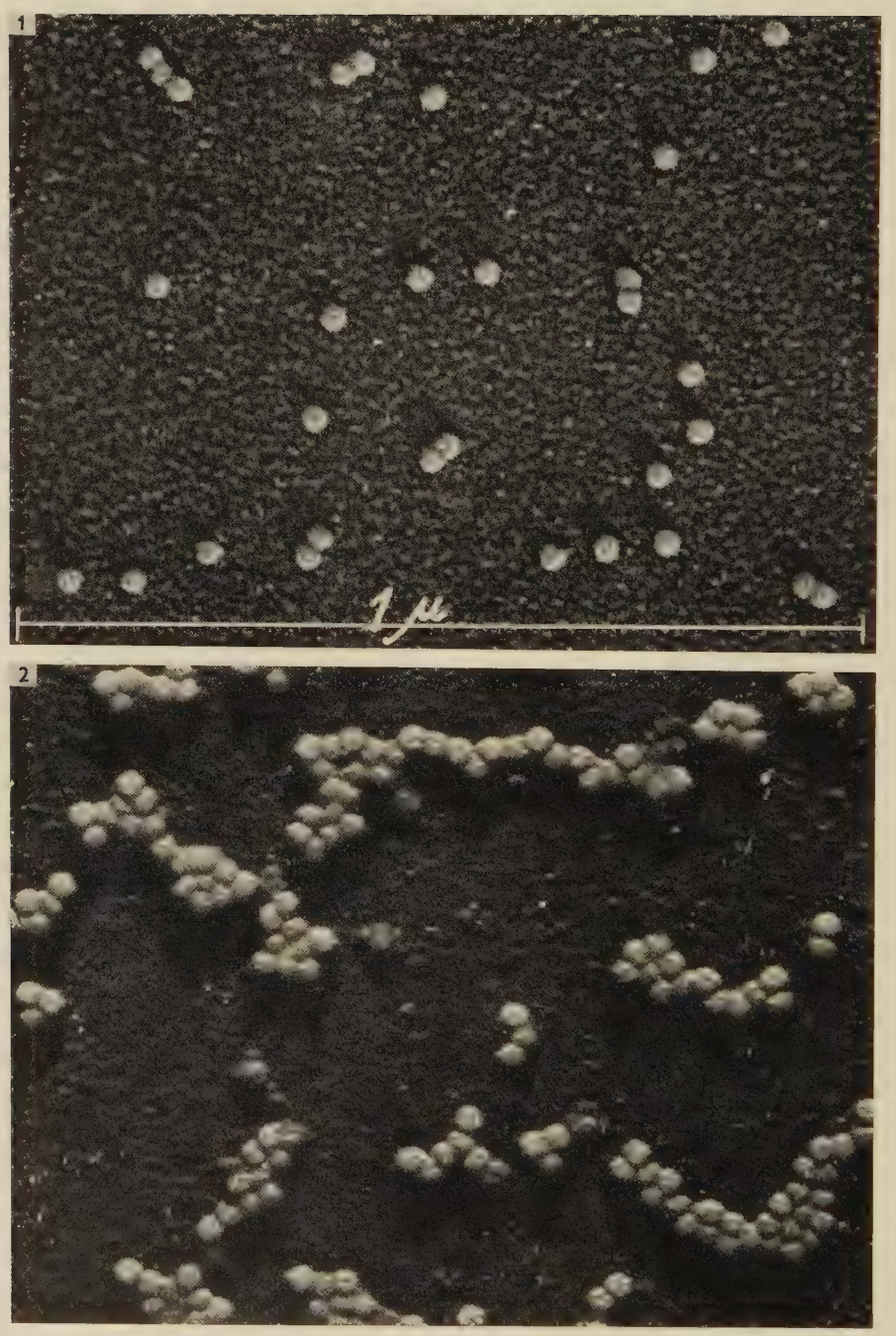

Fig. 1. Spontaneous mouse-encephalomyelitis (strain FA) particles on a beryllium-covered collodion membrane. The particles were dried by heating under a lamp.
Fig. 2. The same kind of preparation as in Fig. 1. The particles were dried by means of phosphorous pentoxide on an ordinary collodion membrane. Both pictures were taken in 1948 with a Siemens microscope at 90 kV, and a primary magnification of 8500.

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